STATEMENT UNDER 37 C.F.R. § 1.607(c)

Pending claims in the present application, *e.g.*, claim 7, correspond exactly or substantially to claims 1-19 of United States Patent No. 6,033,886, issued March 7, 2000, a copy of which is submitted herewith as Exhibit E, cited as reference BT in the Supplemental Information Disclosure Statement filed March 22, 2001.

No fee is believed to be required for this response. However, should any fee be due, please charge the required amount to Pennie & Edmonds LLP Deposit Account No. 16-1150.

Respectfully submitted,

by: Jacqueline Benn
Reg No 43,492

Date May 19, 2003

Laura A. Coruzzi 30,742

Laura A. Coruzzi (Reg. No.)
PENNIE & EDMONDS LLP
1155 Avenue of the Americas
New York, New York 10036-2711
(212) 790-9090

EXHIBIT A
MARKED-UP VERSION OF THE AMENDED PARAGRAPH ON PAGE 1 TO SHOW CHANGES MADE HEREIN

This application is a continuation of U.S. Application Serial No. 09/161,122, filed September 25, 1998, which claims priority benefit to U.S. Provisional Application Serial No. 60/069, 153, filed September 26, 1997, U.S. Provisional Application Serial No. 60/084,133 filed May 1, 1998, and U.S. Provisional Application Serial No. 60/089,207 filed June 12, 1998, and wherein U.S. Application Serial No. 09/161,122, filed September 25, 1998 also claims the right of priority under 35 U.S.C. § 120 to Application No. 08/316,439, filed September 30, 1994, now U.S. Patent No. 5,840,520, issued November 24, 1998.

EXHIBIT B
MARKED-UP VERSION OF THE AMENDED CLAIM TO SHOW CHANGES MADE IN THE PRELIMINARY AMENDMENT OF MAY 19, 2003

8. (amended) The genetically manipulated, infectious virus of Claim 7 [8], the genome of which has been modified to encode a heterologous sequence.

EXHIBIT C
CLAIMS AS THEY WILL BE PENDING IN
U.S. APPLICATION NO.: 09/724,388
UPON ENTRY OF THE AMENDMENT OF MAY 19, 2003

7. A genetically manipulated, infectious virus of the paramyxoviridae family wherein the virus genome comprises a modification wherein the modification is selected from an insertion, substitution or deletion.

8. (amended) The genetically manipulated, infectious virus of Claim 7, the genome of which has been modified to encode a heterologous sequence.

9. The virus of Claim 7 or 8 wherein the virus is a parainfluenza virus.

10. The virus of Claim 7 or 8 wherein the virus is a respiratory syncytial virus.

11. A genetically manipulated, infectious, non-segmented, negative-stranded RNA virus wherein the virus genome comprises a modification wherein the modification is selected from an insertion, substitution or deletion.

12. A vaccine comprising the genetically manipulated viruses of Claims 7, 8, or 11 and a pharmaceutically acceptable carrier.

13. A method for rescuing a genetically manipulated non-segmented negative-stranded RNA virus comprising

introducing into a host cell expressing a heterologous RNA polymerase:

(a) one or more DNA molecules encoding the virus N, P and L proteins operably linked to a polymerase binding site;

(b) a DNA molecule comprising the cDNA of the non-segmented negative stranded RNA virus wherein the cDNA encodes the entire genome of the virus or is modified by the incorporation of a mutation

or a heterologous sequence, and wherein the cDNA is transcribed by a heterologous RNA polymerase; and

isolating the virus produced by the cell.

14. The method of Claim 13 wherein the virus is from the paramyxoviridae family.

15. The method of Claim 14 wherein the virus is parainfluenza virus.

16. The method of Claim 14 wherein the virus is a respiratory syncytial virus

Express Mail No. EL 500 575 856 US First Class Mail ( )
Date Mailed: September 5, 2002
Serial No.: 09/161,122 Filed: September 25, 1998
Inventor: Jin et al.
For: RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES

The stamp of the Patent Office hereon may be taken as an acknowledgment of the date stamped of the following:

(X) Petition to Accept Unintentionally Delayed Claim for Priority under 37 C.F.R. § 1.78(a)(3) (in duplicate);
(X) a Request to Correct Inventorship Under 37 C.F.R. § 1.48(a) (in duplicate);
(X) Statements by Inventors to Be Added Pursuant To 37 C.F.R. § 1.48(a)(2);
(X) a Consent by Assignee for Correction of Inventorship Pursuant to 37 C.F.R. § 1.48(a)(5) with Exhibit 1;
(X) a Declaration for Non-Provisional Patent Application executed by Hong Jin, Roderick Tang, Shengiang Li, Martin Bryant, David Kirkwood Clarke and Peter Palese;
(X) a Sequence Listing in paper and computer-readable form;
(X) a Statement under 37 C.F.R. § 1.825 affirming that the Substitute Sequence Listing and the computer-readable copy thereof are the same and do not include new matter;
(X) Petition for Extension of Time under 37 C.F.R. 1.136(a) for 4 months (in duplicate);
(X) Amendment under 37 C.F.R. § 1.116 with Exhibits A to D;
(X) an Amendment Fee Transmittal Form (in duplicate);
(X) Request for Continued Examination Transmittal (in duplicate);and
(X) Submission of formal drawings (12 sheets of 12 figures).

File No. 7682-045-999 Sender: LAC/JZB/SXM

OIPE JC10 MAY 19 2003 PATENT & TRADEMARK OFFICE

OIPE JC173 SEP 05 2002 PATENT & TRADEMARK OFFICE

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1155 Avenue of the Americas
New York, NY 10109-1441

Exhibit D

Express Mail No.: EL 500 575 856 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: JIN et al.

Application No.: 09/161,122

Filed: September 25, 1998

For: RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES

Group Art Unit: 1642

Examiner: Brumback, B.

Attorney Docket No.:7682-045-999

AMENDMENT UNDER 37 C.F.R. §1.116

Assistant Commissioner for Patents
Box AF
Washington, D.C. 20231

Sir:

In response to the outstanding Final Office Action mailed February 6, 2001, and in accordance with 37 C.F.R. §1.116, please enter the amendments and consider the remarks below intended to put the claims into condition for allowance. Applicants submit concurrently herewith: (1) Petition to Accept Unintentionally Delayed Claim for Priority under 37 C.F.R. § 1.78(a)(3) accompanied by the appropriate fee (in duplicate); (2) a Request to Correct Inventorship Under 37 C.F.R. § 1.48(a) accompanied by the appropriate fee (in duplicate); (3) Statements by Inventors to Be Added Pursuant To 37 C.F.R. § 1.48(a)(2); (4) a Consent by Assignee for Correction of Inventorship Pursuant to 37 C.F.R. § 1.48(a)(5) with Exhibit 1; (5) a Declaration for Non-Provisional Patent Application executed by Hong Jin, Roderick Tang, Shengiang Li, Martin Bryant, David Kirkwood Clarke and Peter Palese; (6) a Sequence Listing in paper and computer-readable form; (7) a Statement under 37 C.F.R. § 1.825 affirming that the Substitute Sequence Listing and the computer-readable copy thereof are the same and do not include new matter; (8) Petition for Extension of Time under 37 C.F.R. 1.136(a) for 4 months accompanied by the appropriate fee (in duplicate); (9) Exhibit

A, an Abstract as required by 37 C.F.R. 1.72(b); (10) Exhibit B a marked-up version of the amended claims wherein brackets indicates deleted matter and underlining indicates inserted matter; (11) Exhibit C, a copy of the claims which will be pending upon entry of this amendment; (12) Exhibit D, a marked-up version of the amended Specification wherein brackets indicates deleted matter and underlining indicates inserted matter; (13) an Amendment Fee Transmittal Form (in duplicate); (14) Request for Continued Examination Transmittal accompanied by the appropriate fee (in duplicate); and (15) under separate transmittal, Submission of formal drawings.

INVENTORSHIP:

Please amend inventorship of the above-captioned application to include Peter Palese and David Kirkwood Clarke.

PRIORITY:

Please amend the claim to priority in the above-identified application to read as follows: the present application claims priority under 35 U.S.C. § 120 to Application Serial No. 08/316,439, filed September 30, 1994 (now U.S. Patent No. 5,840,520); and claims priority to Application Serial No. 60/060,153, filed September 26, 1997; to Application Serial No. 60/084,153, filed May 1, 1998; and Application Serial No. 60/089,207, filed June 12, 1998.

IN THE SPECIFICATION:

Please amend the specification as follows:

On page 1, line 2, please insert the following: --The present application is entitled to and claims right of priority under 35 U.S.C. § 120 to Application No. 08/316,439, filed September 30, 1994, now U.S. Patent No. 5,840,520, issued November 24, 1998; and Application Serial No. 60/060,153, filed September 26, 1997; Application Serial No. 60/084,153, filed May 1, 1998; and Application Serial No. 60/089,207, filed June 12, 1998.--

On page 8, line 15, after 1L-5L, insert --(SEQ ID NOS:1-5)--.

On page 8, line 16, after 1T-9T, insert --(SEQ ID NOS:6-14)--.

On page 9, line 9, after genome, insert --(SEQ ID NOS:15-28)--.

On page 11, line 28, after RSV L protein, insert --(SEQ ID NO:29)--.

On page 11, line 34, after RSV L protein, insert --(SEQ ID NO:29)--.

On page 34, line 13, after AAA C, insert --(SEQ ID NO:1)--.

On page 34, line 15, after TAA CT, insert --(SEQ ID NO:2)--.

On page 34, line 16, after ACT, insert --(SEQ ID NO:3)--.

On page 34, line 18, after TTG TA, insert --(SEQ ID NO:4)--.

On page 34, line 19, after TAC, insert --(SEQ ID NO:5)--.

On page 34, line 22, after AAA TA, insert --(SEQ ID NO:6)--.

On page 34, line 24, after AAT AA, insert --(SEQ ID NO:7)--.

On page 34, line 26, after ACG AG, insert --(SEQ ID NO:8)--.

On page 34, line 28, after ATT A, insert --(SEQ ID NO:9)--.

On page 34, line 29, after ACG A, insert --(SEQ ID NO:10)--.

On page 34, line 31, after CAT AT, insert --(SEQ ID NO:11)--.

On page 34, line 33, after TGA TA, insert --(SEQ ID NO:12)--.

On page 34, line 35, after TTT TA, insert --(SEQ ID NO:13)--.

On page 34, line 36, after CTG CA, insert --(SEQ ID NO:14)--.

On page 37, line 33, after CAGC, insert --(SEQ ID NO:31)--.

On page 37, line 35, after AACA, insert --(SEQ ID NO:32)--.

On page 37, line 37, after CGGT, insert --(SEQ ID NO:33)--.

On page 38, line 2, after CAGC, insert --(SEQ ID NO:34)--.

On page 38, line 4, after AGCT, insert --(SEQ ID NO:35)--.

On page 38, line 6, after CGTTG, insert --(SEQ ID NO:36)--.

On page 38, line 8, after TGGG, insert --(SEQ ID NO:37)--.

On page 38, line 10, after AGGGTCT, insert --(SEQ ID NO:38)--.

On page 38, line 12, after ACTA, insert --(SEQ ID NO:39)--.

On page 38, line 14, after GGTA, insert --(SEQ ID NO:40)--.

On page 38, line 16, after AAG3', insert --(SEQ ID NO:41)--.

On page 51, line 2, before and, insert -- (SEQ ID NO:42)--.

On page 51, line 2, after GG), insert --(SEQ ID NO:43)--.

On page 51, line 17, before and, insert -- (SEQ ID NO:44)--.

On page 51, line 18, after TG), insert --(SEQ ID NO:45)--.

On page 52, line 22, after 3'), insert -- (SEQ ID NO:46)--.

On page 52, line 24, after 3'), insert --(SEQ ID NO:47)--.

On page 68, line 1 please insert the following:

--ABSTRACT

The present invention relates to genetically engineered recombinant Respiratory Syncytial Viruses and viral vectors which contain heterologous genes for use as vaccines. In accordance with the present invention, the recombinant RSV viral vectors and viruses are engineered to contain heterologous genes, including genes of other viruses, pathogens, cellular genes, tumor antigens, or to encode combinations of genes from different strains of RSV.--

IN THE SEQUENCE LISTING

Please amend the sequence listing by entering the replacement sequence listing enclosed herewith pursuant to 37 C.F.R. § 1.825.

IN THE CLAIMS:

Please amend claims 2, 13 and 18 to read as follows:

2. (Amended) An isolated infectious Respiratory Syncytial Virus (RSV) particle containing an RSV RNA comprising a binding site specific for an RNA-directed RNA polymerase operatively linked to an RSV RNA comprising sequences encoding antigenic polypeptides of both RSV-A and RSV-B.

13. (Amended) A vaccine comprising a chimeric Respiratory Syncytial Virus (RSV) the genome of which contains the reverse complement of an mRNA coding sequence operatively linked to a polymerase binding site of an RSV and a pharmaceutically acceptable carrier.

18. (Amended) The vaccine of Claim 13 in which the mRNA coding sequence encodes G and F genes of both Respiratory Syncytial Virus A and Respiratory Syncytial Virus B.

Please add new claims 25-26:

25. (new) The isolated infectious Respiratory Syncytial Virus (RSV) particle of claim 2, wherein the RSV RNA further comprises a L gene mutation.

26. (new) The isolated infectious Respiratory Syncytial Virus (RSV) particle of claim 2, wherein the RSV RNA further comprises a deletion of M2-ORF1 and/or SH-ORF2.

REMARKS

The specification is amended herein to correct technical informalities. Specifically sequence identifiers (SEQ ID NO:) have been inserted in the Brief Description of the Drawings and within the text of the specification wherever nucleotide or amino acid sequences are recited. An abstract has been inserted on the last page of the specification and a copy of the abstract is submitted herewith on a separate sheet as Exhibit A. The proper claim to priority and continuing data have been inserted on page 1, line 2 of the specification. A declaration executed by all the inventors is submitted herewith and establishes the proper priority date of the instant application.

Claims 1-24 are pending. Claims 2, 13, and 18 are under active consideration due to the restriction requirement dated November 17, 1999. Claims 2, 13 and 18 are amended herein to merely correct technical informalities and typographical errors. Claim 2 is amended herein to more particularly point out and distinctly claim that which the applicants regard as the invention. Support for the amendments made herein can be found in the specification page 5, lines 30-37, page 6, lines 1-18, page 7, lines 1-7, and page 15, lines 10-18. Support for claims 2, 3 and 18 can also be found in application Serial No. 08/316,439, filed September 30, 1994, now U.S. Patent No. 5,840,520 which issued on November 24, 1998 on page 10, lines 1-9; page 22, lines 19-27; page 36, lines 19-30; and page 66, line 19 to page 77, line 20.

New claims 25 and 26 have been added to more particularly point out and distinctly claim that which the applicants regard as the invention. Support for new claims 25 and 26 can be found at page 55, line 1 through page 63, line 36 of the instant specification.

A Request to Correct Inventorship under 37 C.F.R. § 1.48 and a Petition to Accept Unintentionally Delayed Claim for Priority under 37 C.F.R. § 1.78(a)(3) are being filed concurrently herewith under separate transmittal.

1. **The Rejections under 35 U.S.C. § 102 are obviated by the <u>amendment made herein and should therefore be withdrawn</u>**

Claims 2, 13, and 18 stand rejected under 35 U.S.C. § 102(b) as being anticipated by Collins et al. (WO97/12032). This rejection is in error and should be withdrawn. The priority date of the instant application, as amended herein, is September 30, 1994 and thus predates the Collins reference of 1997. Thus, Collins et al. (WO97/12032) is not available as art to the instant application and the applicants respectfully submit that the rejection under 35 U.S.C. § 102(b) should be withdrawn.

Claims 2, 13, and 18 stand rejected under 35 U.S.C. § 102(b) as being anticipated by Collins et al., 1995, Proc. Natl. Acad. Sci. 92:11563. This rejection is in error and should be withdrawn. The priority date of the instant application, as amended herein, is September 30, 1994 and thus predates the Collins reference of 1995. Thus, Collins et al., 1995, Proc. Natl. Acad. Sci. 92:11563 is not available as art to the instant application and the applicants respectfully submit that the rejection under 35 U.S.C. § 102(b) should be withdrawn.

2. **The Rejection under 35 U.S.C. § 103 is obviated by the <u>amendment made herein and should therefore be withdrawn</u>**

Claim 18 stands rejected under 35 U.S.C. § 103(a) as being obvious in light of Collins et al., 1995, Proc. Natl. Acad. Sci. 92:11563. This rejection is in error and should be withdrawn. The priority date of the instant application, as amended herein, is September 30, 1994 and thus predates the Collins reference of 1995. Thus, Collins et al., 1995, Proc. Natl. Acad. Sci. 92:11563 is not available as art to the instant application and the applicants respectfully submit that the rejection under 35 U.S.C. § 103(a) should be withdrawn.

CONCLUSION

Applicants respectfully request entry and consideration of the foregoing amendments and remarks. No new matter has been introduced. The claims are believed to be free of the art and patentable. Withdrawal of all the rejections and an allowance are earnestly sought.

Respectfully submitted,

by Jacqueline Benn Reg No. 43,492

Date September 5, 2002

Laura A. Coruzzi 30,742

Laura A. Coruzzi (Reg. No.)

PENNIE & EDMONDS LLP
1155 Avenue of the Americas
New York, New York 10036-2711
(212) 790-9090

EXHIBIT A
ABSTRACT
FILED September 5, 2002
IN U.S. PATENT APPLICATION SERIAL NO. 09/161,122
ATTORNEY DOCKET NO. 7682-045-999

ABSTRACT

The present invention relates to genetically engineered recombinant Respiratory Syncytial Viruses and viral vectors which contain heterologous genes for use as vaccines. In accordance with the present invention, the recombinant RSV viral vectors and viruses are engineered to contain heterologous genes, including genes of other viruses, pathogens, cellular genes, tumor antigens, or to encode combinations of genes from different strains of RSV.

EXHIBIT B
A MARKED UP VERSION OF THE CLAIMS AMENDED IN THE INSTANT AMENDMENT FILED September 5, 2002 IN U.S. PATENT APPLICATION SERIAL NO. 09/161,122 ATTORNEY DOCKET NO. 7682-045-999

---

2. (amended) An isolated infectious Respiratory Syncytial Virus (RSV) [RSV] particle containing an RSV RNA comprising a binding site specific for an RNA-directed RNA polymerase operatively linked to an RSV RNA comprising sequences [which comprises a chimeric RSV antigenome or genome] encoding antigenic polypeptides of both RSV-A and RSV-B.

13. (amended) A vaccine comprising a chimeric Respiratory Syncytial Virus (RSV) [RSV] the genome of which contains the reverse complement of an mRNA coding sequence operatively linked to a polymerase binding site of an RSV and a pharmaceutically acceptable carrier.

18. (amended) The vaccine of Claim 13 in which the mRNA coding sequence encodes G and F genes of both Respiratory Syncytial Virus [RSV] A and Respiratory Syncytial Virus B.

EXHIBIT C
CLAIMS THAT WILL BE PENDING UPON ENTRY OF THE INSTANT AMENDMENT
FILED September 5, 2002
IN U.S. PATENT APPLICATION SERIAL NO. 09/161,122
ATTORNEY DOCKET NO. 7682-045-999

---

2. (amended) An isolated infectious Respiratory Syncytial Virus (RSV) particle containing an RSV RNA comprising a binding site specific for an RNA-directed RNA polymerase operatively linked to an RSV RNA comprising sequences encoding antigenic polypeptides of both RSV-A and RSV-B.

13. (amended) A vaccine comprising a chimeric Respiratory Syncytial Virus (RSV) the genome of which contains the reverse complement of an mRNA coding sequence operatively linked to a polymerase binding site of an RSV and a pharmaceutically acceptable carrier.

18. (amended) The vaccine of Claim 13 in which the mRNA coding sequence encodes G and F genes of both Respiratory Syncytial Virus A and Respiratory Syncytial Virus B.

25. (new) The isolated infectious Respiratory Syncytial Virus (RSV) particle of claim 2, wherein the RSV RNA further comprises a L gene mutation.

26. (new) The isolated infectious Respiratory Syncytial Virus (RSV) particle of claim 2, wherein the RSV RNA further comprises a deletion of M2-ORF1 and/or SH-ORF2.

EXHIBIT D
A MARKED UP VERSION OF PARAGRAPHS IN THE SPECIFICATION AMENDED IN THE INSTANT AMENDMENT FILED September 5, 2002 IN U.S. PATENT APPLICATION SERIAL NO. 09/161,122 ATTORNEY DOCKET NO. 7682-045-999

On page 1, line 2 please insert the following paragraph:

<u>The present application is entitled to and claims right of priority under 35 U.S.C. § 120 to Application No. 08/316,439, filed September 30, 1994, now U.S. Patent No. 5,840,520, issued November 24, 1998; and Application Serial No. 60/060,153, filed September 26, 1997; to Application Serial No. 60/084,153, filed May 1, 1998; and Application Serial No. 60/089,207, filed June 12, 1998.</u>

EXPRESS MAIL NO.: EL 500 575 856 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: JIN et al.

Serial No.: 09/161,122

Group Art Unit: 1642

Filed: September 25, 1998

Examiner: Brumback, B.

For: RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES

Attorney Docket No.: 7682-045-999

PETITION TO ACCEPT UNINTENTIONALLY DELAYED CLAIM FOR PRIORITY UNDER 37 C.F.R. § 1.78(a)(3)

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Pursuant to 37 C.F.R. § 1.78(a)(3), Applicants hereby petition the Commissioner to accept the unintentionally delayed claim for priority. Applicants submit an amendment under 37 C.F.R. § 1.116 concurrently herewith.

The delay in claiming priority was unintentional. In particular, inventors David Kirkwood Clarke and Peter Palese who are the only inventors on the prior application have inadvertently been omitted from the above-identified application. A request to correct inventorship under 37 C.F.R. § 1.48(a) is concurrently herewith. Applicants note that the delay in claiming priority was caused by the lack of co-inventorship between the prior application and the above-identified application. The lack of co-inventorship occurred in error as the inventors David Kirkwood Clarke and Peter Palese who are the only inventors on the prior application were inadvertently omitted from the above-identified application.

Applicants hereby submit the fee of $1,280.00 set forth in 37 C.F.R. § 1.17(t). Please charge the required surcharge to Pennie & Edmonds LLP Deposit Account No. 16-1150. The

Commissioner is hereby authorized to charge any deficiency of any fee, or surcharge, or other payment to Pennie & Edmonds LLP Deposit Account No. 16-1150.

The Petitioners request that the unintentionally delayed claim for priority be entered in the instant application.

Respectfully submitted,

by: Jacqueline Benn Reg No. 43,492

Laura A. Coruzzi 30,742

Date: September 5, 2002

Laura A. Coruzzi (Reg. No.)
PENNIE & EDMONDS LLP
1155 Avenue of the Americas
New York, NY 10036-2711
Telephone: (212) 790-9090

Enclosures

Express Mail No.: EL 500 575 856 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: JIN et al.

Serial No.: 09/161,122

Filed: September 25, 1998

For: RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES

Group Art Unit: 1642

Examiner: Brumback, B.

Attorney Docket No.: 7682-045-999

REQUEST TO CORRECT INVENTORSHIP UNDER 37 C.F.R. § 1.48 (a)

Assistant Commissioner for Patents
U.S. Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202

SIR:

It is respectfully requested that the inventorship of the above-identified patent application be corrected under 37 C.F.R. § 1.48(a) to further include the name of David Kirkwood Clarke, whose residence is 3205 Whispering Hills, Chester, New York 10918, and Peter Palese, whose residence is 414 Highwood Avenue, Leonia, New Jersey 07065, as co-inventors of the claims in the instant patent application. Applicants concurrently submit herewith (1) a Statement in Support of Correction of Inventorship; (2) an executed Declaration by the actual inventors; and (3) a Consent by Assignee to Correction of Inventorship.

The name of David Kirkwood Clarke and Peter Palese were omitted in the instant patent application through error and without any deceptive intention on the part of the omitted inventor. Thus, the inventorship of the claims in the instant patent application should be corrected to include the names of David Kirkwood Clarke and Peter Palese.

Pursuant to 37 C.F.R. § 1.48(a) and § 1.17(i), it is believed that a fee of $130.00 is due for submission of this request. Please charge the required fee to Pennie and Edmonds LLP Deposit Account Number 16-1150; a duplicate of this sheet is enclosed.

Respectfully submitted,

by Jacqueline Benn Reg No. 43,492

Date: September 5, 2002

Laura A. Coruzzi 30,742

Laura A. Coruzzi (Reg. No.)

PENNIE & EDMONDS LLP
1155 Avenue of the Americas
New York, NY 10036-2711
Telephone: (212) 790-9090

Enclosures

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Jin et al.

Serial No.: 09/161,122

Filed: September 25, 1998

For: RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES

Group Art Unit: 1642

Examiner: Brumback, B.

Attorney Docket No.: 7682-045-999

STATEMENT BY THE INVENTOR TO BE ADDED PURSUANT TO 37 C.F.R. §1.48 (a)(2)

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Peter M. Palese, have reviewed and understand the content of the Petition for Correction of Inventorship Under 37 C.F.R. §1.48(a) submitted concurrently herewith to amend the above-identified application to correctly name all of the inventors. I hereby state that my name was inadvertently omitted, without deceptive intent, as a co-inventor of the currently claimed subject matter of the above-identified patent application. There was no deceptive intent on my part in the omission of my name as a co-inventor.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that I make these statements with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

8/29/02
Date

Peter M. Palese

NY2 - 1282485.1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Jin et al.

Serial No.: 09/161,122

Filed: September 25, 1998

For: RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES

Group Art Unit: 1642

Examiner: Brumback, B.

Attorney Docket No.: 7682-045-999

STATEMENT BY THE INVENTOR TO BE ADDED PURSUANT TO 37 C.F.R. §1.48 (a)(2)

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, David Kirkwood Clarke, have reviewed and understand the content of the Petition for Correction of Inventorship Under 37 C.F.R. §1.48(a) submitted concurrently herewith to amend the above-identified application to correctly name all of the inventors. I hereby state that my name was inadvertently omitted, without deceptive intent, as a co-inventor of the currently claimed subject matter of the above-identified patent application. There was no deceptive intent on my part in the omission of my name as a co-inventor.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that I make these statements with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

8.22.02
Date

David K. Clarke
David Kirkwood Clarke

NY2 - 1282475.1

Express Mail No.: EL 500 575 856 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: JIN et al.

Serial No.: 09/161,122

Filed: September 25, 1998

For: RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES

Group Art Unit: 1642

Examiner: Brumback, B.

Attorney Docket No.: 7682-045-999

STATEMENT UNDER 37 C.F.R. §§ 1.825(a) AND 1.825(b)

Assistant Commissioner for Patents
Washington, D.C. 20231

SIR:

Applicants submit herewith copies of the Substitute Sequence Listing in paper and computer-readable forms.

Applicants submit herewith a statement that the paper and computer-readable copies of the Substitute Sequence Listing, submitted in accordance with 37 C.F.R. § 1.825 on even date herewith, are the same. Applicants further submit herewith a statement that the paper and computer-readable copies of the Substitute Sequence Listing, submitted in accordance with 37 C.F.R. § 1.825 on even date herewith, do not include new matter. Accordingly, Applicants respectfully request that the Substitute Sequence Listing be entered in the instant application.

Respectfully submitted,

by Jacqueline Benn Reg No. 43,492

Date September 5, 2002

Laura A. Coruzzi 30,742

Laura A. Coruzzi (Reg. No.)

PENNIE & EDMONDS LLP
1155 Avenue of the Americas
New York, New York 10036-2711
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NY2 - 1349736.1

PTO/SB/30 (5/2000)
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U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

REQUEST FOR CONTINUED EXAMINATION (RCE) TRANSMITTAL

Subsection (b) of 35 U.S.C. § 132, effective on May 29, 2000, provides for continued examination of an utility or plant application filed on or after June 8, 1995.
See The American Inventors Protection Act of 1999 (AIPA).

Express Mail No.	EL 500 575 856 US
Application Number	09/161,122
Filing Date	September 25, 1998
First Named Inventor	JIN, H.
Group Art Unit	1642
Examiner Name	Brumback, B.
Attorney Docket Number	7682-045-999

This is a Request for Continued Examination (RCE) under 37 C.F.R. § 1.114 of the above-identified application.
NOTE: 37 C.F.R. § 1.114 is effective on May 29, 2000. If the above-identified application was filed prior to May 29, 2000, applicant may wish to consider filing a continued prosecution application (CPA) under 37 C.F.R. § 1.53 (d) (PTO/SB/29) instead of a RCE to be eligible for the patent term adjustment provisions of the AIPA. See Changes to Application Examination and Provisional Application Practice, Interim Rule, 65 Fed. Reg. 14865 *(Mar. 20, 2000), 1233 Off. Gaz. Pat. Office 47 (Apr. 11, 2000), which established RCE practice.*

1. **Submission required under 37 C.F.R. § 1.114**
   - a. ☐ Previously submitted
     - i. ☐ Consider the amendment(s)/reply under 37 C.F.R. § 1.116 previously filed on ____ (Any unentered amendment(s) referred to above will be entered).
     - ii. ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on ____
     - iii. ☐ Other ____
   - b. ☒ Enclosed
     - i. ☒ Amendment/Reply
     - ii. ☐ Affidavit(s)/Declaration(s)
     - iii. ☐ Information Disclosure Statement (IDS)
     - iv. ☒ Other Petition for Extension of Time for 4 months; Petition to Accept Unintentionally Delayed Claim for Priority under 37 C.F.R. 1.78(a)(3); Request to Correct Inventorship under 37 C.F.R. 1.48(a); Submission of Substitute Sequence Listing; Submission of Formal Drawings
2. **Miscellaneous**
   - a. ☐ Suspension of action on the above-identified application is requested under 37 C.F.R. § 1.103(c) for a period of ____ months. (Period of suspension shall not exceed 3 months; Fee under 37 C.F.R. § 1.17(i) required)
   - b. ☐ Other ____
3. **Fees** The RCE fee under 37 C.F.R. § 1.17(e) is required by 37 C.F.R. § 1.114 when the RCE is filed
   - a. ☒ The Director is hereby authorized to charge the following fees, or credit any overpayments, to Pennie & Edmonds LLP Deposit Account No. 16-1150:
     - i. ☒ RCE fee required under 37 C.F.R. § 1.17(e), estimated to be $ 740.00
     - ii. ☐ Extension of time fee required under 37 C.F.R. §§ 1.136 and 1.17, estimated to be $__ for a __ month extension, the request for which is being made herewith
     - iii. ☐ Other __
   - b. ☐ Check in the amount of $___ enclosed
   - c. ☐ Payment by credit card (Form PTO-2038 enclosed)

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Name (Print/Type)	Laura A. Coruzzi by Jacqueline B[...]	Registration No. (Attorney/Agent)	30,742
Signature	Laura A. Coruzzi Reg No. 43,492	Date	September 5, 2002

CERTIFICATE OF MAILING OR TRANSMISSION

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner For Patents, Box RCE, Washington, DC 20231, or by facsimile transmitted to fax no. 1-703- to the U.S. Patent and Trademark Office on the date indicated below.

Name (Print/Type)		Registration No. (Attorney/Agent)	
Signature		Date	

Express Mail No.: EL 500 575 856 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: JIN et al.

Application No.: 09/161,122

Filed: September 25, 1998

For: RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES

Group Art Unit: 1642

Examiner: Brumback, B.

Attorney Docket No.: 7682-045-999

FEE TRANSMITTAL SHEET

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

The fee required to be filed with the accompanying amendment of even date herewith concerning the above-identified application has been estimated to be $0.

The claim amendment fee has been estimated as shown below:

	(Col. 1)		(Col. 2)		(Col. 3)	SMALL ENTITY			OTHER THAN A SMALL ENTITY	
	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NO PREVIOUSLY PAID FOR		PRESENT EXTRA	RATE	ADDIT. FEE	OR	RATE	ADDIT. FEE
TOTAL	5	MINUS	27	=	0	× 9	$		× 18	$ 0.00
INDEP.	2	MINUS	7	=	0	× 42	$		× 84	$ 0.00
☐ FIRST PRESENTATION OF MULTIPLE DEP. CLAIM						140	$		280	$
						TOTAL	$	OR	TOTAL	$ 0.00

Please charge the required fee to Pennie & Edmonds LLP Deposit Account No. 16-1150. A copy of this sheet is enclosed.

Respectfully submitted, by: Jacqueline Benn Reg No. 43,492

Laura A. Coruzzi 30,742

Laura A. Coruzzi (Reg. No.)

Date September 5, 2002

PENNIE & EDMONDS LLP
1155 Avenue of Americas
New York, N.Y. 10036-2711
(212) 790-9090

Enclosure

Express Mail No.: EL 500 575 856 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: JIN et al.

Serial No.: 09/161,122

Filed: September 25, 1998

For: RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES

Art Unit: 1642

Examiner: Brumback, B.

Attorney Docket No.: 7682-045-999

PETITION FOR EXTENSION OF TIME UNDER 37 CFR § 1.136(a)

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

It is respectfully requested that the time for response to the Office Action dated March 7, 2002, be extended for a period of 4 month(s) from May 7, 2002 to and including September 7, 2002.

The fee for this extension is estimated to be $1,440.00. Please charge the required fee to Pennie & Edmonds LLP Deposit Account No. 16-1150. A copy of this sheet is enclosed.

Respectfully submitted, by: Jacqueline Benn Reg No. 43,492

Laura A. Coruzzi 30,742 (Reg. No.)

Date September 5, 2002

Laura A. Coruzzi
PENNIE & EDMONDS LLP
1155 Avenue of the Americas
New York, N.Y. 10036-2711
(212) 790-9090

NY2 - 1349740.1

DECLARATION FOR NON-PROVISIONAL PATENT APPLICATION*

below named inventor, I hereby declare that:

esidence, post office address and citizenship are as stated below at 201 et seq. beneath my name.

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RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES

for which a patent application:
attached hereto and includes amendment(s) filed on *(if applicable)*
as filed in the United States on September 25, 1998 as Application No. 09/161,122 *(for declaration not accompanying application)*
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as filed as PCT international Application No. on and was amended under PCT Article 19 on *(if applicable)*

reby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any :ndment referred to above.

.knowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, 56.

:reby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's tificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that :he application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			YES ☐ NO ☐
			YES ☐ NO ☐
			YES ☐ NO ☐

hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

PROVISIONAL APPLICATION NUMBER	FILING DATE
60/060,153	September 26, 1997
60/084,133	May 1, 1998
60/089,207	June 12, 1998

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information known to me which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

NON-PROVISIONAL APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED
08/316,439	September 30, 1994	✔		

* for use only when the application is assigned to a company, partnership or other organization.

ereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are lieved to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are nishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements ay jeopardize the validity of the application or any patent issuing thereon.

	FULL NAME OF INVENTOR	LAST NAME: JIN	FIRST NAME: Hong	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: Cupertino	STATE OR FOREIGN COUNTRY: California	COUNTRY OF CITIZENSHIP: People's Republic of China USA.	
	POST OFFICE ADDRESS	STREET: 22385 Santa Paula Avenue	CITY: Cupertino	STATE OR COUNTRY: California	ZIP CODE: 95014
		SIGNATURE OF INVENTOR 201		DATE: 8/30/02	
202	FULL NAME OF INVENTOR	LAST NAME: TANG	FIRST NAME: Roderick	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: San Carlos	STATE OR FOREIGN COUNTRY: California	COUNTRY OF CITIZENSHIP: Malaysia	
	POST OFFICE ADDRESS	STREET: 730 Chestnut Street	CITY: San Carlos	STATE OR COUNTRY: California	ZIP CODE: 94070
		SIGNATURE OF INVENTOR 202		DATE: 30 Aug 02	
203	FULL NAME OF INVENTOR	LAST NAME: LI	FIRST NAME: Shengqiang	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: Los Altos	STATE OR FOREIGN COUNTRY: California	COUNTRY OF CITIZENSHIP: People's Republic of China	
	POST OFFICE ADDRESS	STREET: 718 Terrace Court	CITY: Los Altos	STATE OR COUNTRY: California	ZIP CODE: 94024
		SIGNATURE OF INVENTOR 203		DATE:	
204	FULL NAME OF INVENTOR	LAST NAME: BRYANT	FIRST NAME: Martin	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: Carlisle	STATE OR FOREIGN COUNTRY: Massachusetts	COUNTRY OF CITIZENSHIP: United States of America	
	POST OFFICE ADDRESS	STREET: 65 Hickory Lane	CITY: Carlisle	STATE OR COUNTRY: Massachusetts	ZIP CODE: 01741
		SIGNATURE OF INVENTOR 204		DATE:	
205	FULL NAME OF INVENTOR	LAST NAME: CLARKE	FIRST NAME: David	MIDDLE NAME: Kirkwood	
	RESIDENCE & CITIZENSHIP	CITY: Chester	STATE OR FOREIGN COUNTRY: New York	COUNTRY OF CITIZENSHIP: United Kingdom/United States of America	
	POST OFFICE ADDRESS	STREET: 30 Whispering Hills	CITY: Chester	STATE OR COUNTRY: New York	ZIP CODE: 10918
		SIGNATURE OF INVENTOR 203		DATE:	

DECLARATION FOR NON-PROVISIONAL PATENT APPLICATION*

ı below named inventor, I hereby declare that:

residence, post office address and citizenship are as stated below at 201 et seq. beneath my name.

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RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES

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s attached hereto and includes amendment(s) filed on *(if applicable)*
vas filed in the United States on September 25, 1998 as Application No. 09/161,122 *(for declaration not accompanying application)*
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ıereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's rtificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION				
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED	
			YES ☐	NO ☐
			YES ☐	NO ☐
			YES ☐	NO ☐

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

PROVISIONAL APPLICATION NUMBER	FILING DATE
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I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information known to me which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

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hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

201	FULL NAME OF INVENTOR	LAST NAME JIN	FIRST NAME Hong	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY Cupertino	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP People's Republic of China	
	POST OFFICE ADDRESS	STREET 22385 Santa Paula Avenue	CITY Cupertino	STATE OR COUNTRY California	ZIP CODE 95014
		SIGNATURE OF INVENTOR 201		DATE	
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	RESIDENCE & CITIZENSHIP	CITY San Carlos	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP Malaysia	
	POST OFFICE ADDRESS	STREET 730 Chestnut Street	CITY San Carlos	STATE OR COUNTRY California	ZIP CODE 94070
		SIGNATURE OF INVENTOR 202		DATE	
203	FULL NAME OF INVENTOR	LAST NAME LI	FIRST NAME Shengqiang	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY Los Altos	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP People's Republic of China	
	POST OFFICE ADDRESS	STREET 718 Terrace Court	CITY Los Altos	STATE OR COUNTRY California	ZIP CODE 94024
		SIGNATURE OF INVENTOR 203 Shengqiang Li		DATE 8/29/2002	
204	FULL NAME OF INVENTOR	LAST NAME BRYANT	FIRST NAME Martin	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY Carlisle	STATE OR FOREIGN COUNTRY Massachusetts	COUNTRY OF CITIZENSHIP United States of America	
	POST OFFICE ADDRESS	STREET 65 Hickory Lane	CITY Carlisle	STATE OR COUNTRY Massachusetts	ZIP CODE 01741
		SIGNATURE OF INVENTOR 204		DATE	
205	FULL NAME OF INVENTOR	LAST NAME CLARKE	FIRST NAME David	MIDDLE NAME Kirkwood	
	RESIDENCE & CITIZENSHIP	CITY Chester	STATE OR FOREIGN COUNTRY New York	COUNTRY OF CITIZENSHIP United Kingdom/United States of America	
	POST OFFICE ADDRESS	STREET 30 Whispering Hills	CITY Chester	STATE OR COUNTRY New York	ZIP CODE 10918
		SIGNATURE OF INVENTOR 203		DATE	

206

FULL NAME OF INVENTOR	LAST NAME PALESE	FIRST NAME Peter	MIDDLE NAME	
RESIDENCE & CITIZENSHIP	CITY Leonia	STATE OR FOREIGN COUNTRY New Jersey	COUNTRY OF CITIZENSHIP United States of America	
POST OFFICE ADDRESS	STREET 414 Highwood Avenue	CITY Leonia	STATE OR COUNTRY New Jersey	ZIP CODE 07065
	SIGNATURE OF INVENTOR 203		DATE	

DECLARATION FOR NON-PROVISIONAL PATENT APPLICATION*

ı below named inventor, I hereby declare that:

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			YES ☐	NO ☐
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

No.					
201	FULL NAME OF INVENTOR	LAST NAME Jin	FIRST NAME Hong	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY Cupertino	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP People's Republic of China	
	POST OFFICE ADDRESS	STREET 22385 Santa Paula Avenue	CITY Cupertino	STATE OR COUNTRY California	ZIP CODE 95014
		SIGNATURE OF INVENTOR 201		DATE	
202	FULL NAME OF INVENTOR	LAST NAME Tang	FIRST NAME Roderick	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY San Carlos	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP Malaysia	
	POST OFFICE ADDRESS	STREET 730 Chestnut Street	CITY San Carlos	STATE OR COUNTRY California	ZIP CODE 94070
		SIGNATURE OF INVENTOR 202		DATE	
203	FULL NAME OF INVENTOR	LAST NAME Li	FIRST NAME Shengiang	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY Palo Alto	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP People's Republic of China	
	POST OFFICE ADDRESS	STREET 4290 Wilkie Way	CITY Palo Alto	STATE OR COUNTRY California	ZIP CODE 94306
		SIGNATURE OF INVENTOR 203		DATE	
204	FULL NAME OF INVENTOR	LAST NAME Bryant	FIRST NAME Martin	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY Carlisle	STATE OR FOREIGN COUNTRY Massachusetts	COUNTRY OF CITIZENSHIP United States of America	
	POST OFFICE ADDRESS	STREET 65 Hickory Lane	CITY Carlisle	STATE OR COUNTRY Massachusetts	ZIP CODE 01741
		SIGNATURE OF INVENTOR 204 Martin Bryant		DATE 12 Dec 2001	
205	FULL NAME OF INVENTOR	LAST NAME Palese	FIRST NAME Peter	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY Leonia	STATE OR FOREIGN COUNTRY New Jersey	COUNTRY OF CITIZENSHIP United States of America	
	POST OFFICE ADDRESS	STREET 414 Highwood Avenue	CITY Leonia	STATE OR COUNTRY New Jersey	ZIP CODE 07605
		SIGNATURE OF INVENTOR 205		DATE	

206

FULL NAME OF INVENTOR	LAST NAME PALESE	FIRST NAME Peter	MIDDLE NAME	
RESIDENCE & CITIZENSHIP	CITY Leonia	STATE OR FOREIGN COUNTRY New Jersey	COUNTRY OF CITIZENSHIP United States of America	
POST OFFICE ADDRESS	STREET 414 Highwood Avenue	CITY Leonia	STATE OR COUNTRY New Jersey	ZIP CODE 07065
	SIGNATURE OF INVENTOR 203		DATE	

PENNIE & EDMONDS LLP DOCKET NO. 7682-045-999

206	FULL NAME OF INVENTOR	LAST NAME Clarke	FIRST NAME David	MIDDLE NAME Kirkwood	
	RESIDENCE & CITIZENSHIP	CITY Chester	STATE OR FOREIGN COUNTRY New York	COUNTRY OF CITIZENSHIP United Kingdom/ United States	
	POST OFFICE ADDRESS	STREET 30 Whispering Hills	CITY Chester	STATE OR COUNTRY New York	ZIP CODE 10918
		SIGNATURE OF INVENTOR 205		DATE	

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NY2 - 1282667.1

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	RESIDENCE & CITIZENSHIP	CITY San Carlos	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP Malaysia	
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		SIGNATURE OF INVENTOR 202		DATE	
203	FULL NAME OF INVENTOR	LAST NAME LI	FIRST NAME Shengqiang	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY Los Altos	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP People's Republic of China	
	POST OFFICE ADDRESS	STREET 718 Terrace Court	CITY Los Altos	STATE OR COUNTRY California	ZIP CODE 94024
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205	FULL NAME OF INVENTOR	LAST NAME CLARKE	FIRST NAME David	MIDDLE NAME Kirkwood	
	RESIDENCE & CITIZENSHIP	CITY Chester	STATE OR FOREIGN COUNTRY New York	COUNTRY OF CITIZENSHIP United Kingdom/United States of America	
	POST OFFICE ADDRESS	STREET 30 Whispering Hills	CITY Chester	STATE OR COUNTRY New York	ZIP CODE 10918
		SIGNATURE OF INVENTOR 203		DATE	

PENNIE & EDMONDS LLP DOCKET NO. 7682-045-999

FULL NAME OF INVENTOR	LAST NAME PALESE	FIRST NAME Peter	MIDDLE NAME
RESIDENCE & CITIZENSHIP	CITY Leonia	STATE OR FOREIGN COUNTRY New Jersey	COUNTRY OF CITIZENSHIP United States of America
POST OFFICE ADDRESS	STREET 414 Highwood Avenue	CITY Leonia	STATE OR COUNTRY New Jersey · ZIP CODE 07065
	SIGNATURE OF INVENTOR 203	Pol Pal	DATE 8/29/02

NY2 - 1282667.1

DECLARATION FOR NON-PROVISIONAL PATENT APPLICATION*

s a below named inventor, I hereby declare that:

ly residence, post office address and citizenship are as stated below at 201 et seq. beneath my name.

believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names re listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES

nd for which a patent application:
is attached hereto and includes amendment(s) filed on *(if applicable)*
was filed in the United States on September 25, 1998 as Application No. 09/161,122 *(for declaration not accompanying application)*
with amendment(s) filed on even date herewith *(if applicable)*
was filed as PCT international Application No. on and was amended under PCT Article 19 on *(if applicable)*

hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			YES ☐ NO ☐
			YES ☐ NO ☐
			YES ☐ NO ☐

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

PROVISIONAL APPLICATION NUMBER	FILING DATE
60/060,153	September 26, 1997
60/084,133	May 1, 1998
60/089,207	June 12, 1998

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information known to me which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

NON-PROVISIONAL APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED
08/316,439	September 30, 1994	✔		

* for use only when the application is assigned to a company, partnership or other organization.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

201	FULL NAME OF INVENTOR	LAST NAME Jin	FIRST NAME Hong	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY Cupertino	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP People's Republic of China	
	POST OFFICE ADDRESS	STREET 22385 Santa Paula Avenue	CITY Cupertino	STATE OR COUNTRY California	ZIP CODE 95014
		SIGNATURE OF INVENTOR 201		DATE	
202	FULL NAME OF INVENTOR	LAST NAME Tang	FIRST NAME Roderick	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY San Carlos	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP Malaysia	
	POST OFFICE ADDRESS	STREET 730 Chestnut Street	CITY San Carlos	STATE OR COUNTRY California	ZIP CODE 94070
		SIGNATURE OF INVENTOR 202		DATE	
203	FULL NAME OF INVENTOR	LAST NAME Li	FIRST NAME Shengiang	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY Palo Alto	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP People's Republic of China	
	POST OFFICE ADDRESS	STREET 4290 Wilkie Way	CITY Palo Alto	STATE OR COUNTRY California	ZIP CODE 94306
		SIGNATURE OF INVENTOR 203		DATE	
204	FULL NAME OF INVENTOR	LAST NAME Bryant	FIRST NAME Martin	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY Carlisle	STATE OR FOREIGN COUNTRY Massachusetts	COUNTRY OF CITIZENSHIP United States of America	
	POST OFFICE ADDRESS	STREET 65 Hickory Lane	CITY Carlisle	STATE OR COUNTRY Massachusetts	ZIP CODE 01741
		SIGNATURE OF INVENTOR 204		DATE	
205	FULL NAME OF INVENTOR	LAST NAME Palese	FIRST NAME Peter	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY Leonia	STATE OR FOREIGN COUNTRY New Jersey	COUNTRY OF CITIZENSHIP United States of America	
	POST OFFICE ADDRESS	STREET 414 Highwood Avenue	CITY Leonia	STATE OR COUNTRY New Jersey	ZIP CODE 07605
		SIGNATURE OF INVENTOR 205		DATE	

FULL NAME OF INVENTOR	LAST NAME Clarke	FIRST NAME David	MIDDLE NAME Kirkwood	
RESIDENCE & CITIZENSHIP	CITY Chester	STATE OR FOREIGN COUNTRY New York	COUNTRY OF CITIZENSHIP United Kingdom/ United States	
POST OFFICE ADDRESS	STREET 30 Whispering Hills	CITY Chester	STATE OR COUNTRY New York	ZIP CODE 10918
	SIGNATURE OF INVENTOR 205 David K Clarke		DATE 12-14-01	

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: JIN et al.

Serial No.: 09/161,122

Filed: September 25, 1998

For: RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES

Group Art Unit: 1642

Examiner: Brumback, B.

Attorney Docket No.: 7682-045-999

CONSENT BY ASSIGNEE FOR CORRECTION OF INVENTORSHIP PURSUANT TO 37 C.F.R. § 1.48(a)(5)

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

MEDIMMUNE VACCINES, Inc., having an office for transaction of business at 297 North Bernardo Avenue, Mountain View, California 94043, as an assignee of right, title and interest in, to and under the invention and U.S. patent application Serial No. 09/161,122, filed September 25, 1998, entitled "RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES", hereby consents to amendment of the application to name the following actual inventors: Hong Jin, Roderick Tang, Shengqiang Li, Martin Bryant, David Clarke, and Peter Palese.

An Assignment of the application to AVIRON, Inc. executed by Hong Jin, Roderick Tang, Shengqiang Li, and Martin Bryant was recorded with the U.S. Patent & Trademark Office on April 16, 2001, on Reel 011706 at frame 0529. Attached hereto as Exhibit 1 is a Certificate of Amendment to Amended and Restated Certificate of Incorporation certifying that the name of the corporation previously known as AVIRON, Inc. has been amended to MEDIMMUNE VACCINES, Inc.

The undersigned is empowered to act on behalf of the assignee.

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and

further, that these statements are made with the knowledge that willful false statements, and the like so made, are punishable by fine or imprisonment, or both, under Section 1001, Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 9/3/02

Timothy R. Pearson
Signature

Timothy R. Pearson
Name of Signatory on behalf of
MEDIMMUNE VACCINES, Inc.

Vice President, Treasurer & Secretary
Title of Signatory

Delaware

The First State

I, HARRIET SMITH WINDSOR, SECRETARY OF STATE OF THE STATE OF DELAWARE, DO HEREBY CERTIFY THE ATTACHED IS A TRUE AND CORRECT COPY OF THE CERTIFICATE OF OWNERSHIP OF "AVIRON", FILED IN THIS OFFICE ON THE FIFTEENTH DAY OF JANUARY, A.D. 2002, AT 4 O'CLOCK P.M.

AND I DO HEREBY FURTHER CERTIFY THAT THE ANNUAL REPORTS HAVE BEEN FILED TO DATE.

AND I DO HEREBY FURTHER CERTIFY THAT THE FRANCHISE TAXES HAVE BEEN PAID TO DATE.

Harriet Smith Windsor

Harriet Smith Windsor, Secretary of State

2600915 8100

020188821

AUTHENTICATION: 1681841

DATE: 03-21-02

STATE OF DELAWARE
SECRETARY OF STATE
DIVISION OF CORPORATIONS
FILED 04:00 PM 01/15/2002
020028088 – 2600915

CERTIFICATE OF OWNERSHIP AND MERGER

OF

APPLE MERGER CORP.

WITH AND INTO

AVIRON

Under Section 253
of the Delaware General Corporation Law

Apple Merger Corp., a Delaware corporation (the "Corporation"), hereby certifies as follows:

FIRST: The Corporation was incorporated on November 29, 2001, pursuant to the Delaware General Corporation Law (the "DGCL").

SECOND: The Corporation is the owner of at least ninety percent of the outstanding shares of common stock of Aviron. The shares of common stock constitute the only outstanding shares of capital stock of Aviron.

THIRD: The following is a copy of the resolutions duly adopted as of January 15th, 2002 by the Written Consent of the Board of Directors of the Corporation with respect to the merger of the Corporation with and into Aviron:

> "RESOLVED, that the Corporation be merged (the "Merger") with and into Aviron, with Aviron as the surviving corporation, on the terms and subject to the conditions set forth in the Agreement and Plan of Merger (the "Merger Agreement") dated as of December 2, 2001 among MedImmune, Inc. ("Parent"), the Corporation and Aviron, and the Merger is hereby approved; and further
>
> RESOLVED, that at the effective time of the Merger:
>
> 1. Each issued and outstanding share of capital stock of the Corporation shall be converted into and become one validly issued, fully paid and nonassessable share of common stock of Aviron, as the surviving corporation.
>
> 2. Each share of common stock (the "Shares") of Aviron that is owned by Parent, the Corporation or Aviron shall

automatically be canceled and retired and shall cease to exist, and no consideration shall be delivered in exchange therefor.

3. Each issued and outstanding Share (other than any shares to be canceled in accordance with 2, above, and other than Shares held by stockholders who perfect appraisal rights under Delaware law) shall be converted into the right to receive 1.075 validly issued, fully paid and nonassessable shares of common stock (the "Parent Shares") of Parent. Notwithstanding the foregoing, each holder of Shares exchanged pursuant to the Merger who would otherwise have been entitled to receive a fraction of a Parent Share (after taking into account all certificates representing Shares delivered by such holder) shall receive, in lieu thereof, cash (without interest) in an amount equal to such fractional part of a Parent Share multiplied by the closing price for a Parent Share as reported in the New York City edition of The Wall Street Journal (or, if not reported thereby, any other authoritative source) on the date prior to the date of the Merger.

FOURTH: The Merger has been approved by MedImmune, Inc., the sole stockholder of the Corporation, by written consent in lieu of a meeting pursuant to Section 228 of the DGCL.

IN WITNESS WHEREOF, the undersigned has duly executed this Certificate of Ownership and Merger this 15th day of January, 2002.

APPLE MERGER CORP.

By: David M. Mott
Name: David M. Mott
Title: Chief Executive Officer

Delaware

The First State

I, HARRIET SMITH WINDSOR, SECRETARY OF STATE OF THE STATE OF DELAWARE, DO HEREBY CERTIFY THE ATTACHED IS A TRUE AND CORRECT COPY OF THE RESTATED CERTIFICATE OF "AVIRON", FILED IN THIS OFFICE ON THE FIFTEENTH DAY OF JANUARY, A.D. 2002, AT 4:01 O'CLOCK P.M.

AND I DO HEREBY FURTHER CERTIFY THAT THE ANNUAL REPORTS HAVE BEEN FILED TO DATE.

AND I DO HEREBY FURTHER CERTIFY THAT THE FRANCHISE TAXES HAVE BEEN PAID TO DATE.

Harriet Smith Windsor

Harriet Smith Windsor, Secretary of State

2600915 8100

020188822

AUTHENTICATION: 1681842

DATE: 03-21-02

STATE OF DELAWARE
DIVISION OF CORPORATIONS
FILED 04:01 PM 01/15/2002
020028094 – 2600915

AMENDED AND RESTATED

CERTIFICATE OF INCORPORATION

OF

AVIRON

Pursuant to Sections 242 and 245 of the General Corporation Law of the State of Delaware

Aviron, a corporation organized and existing under the laws of the State of Delaware (the "Corporation"), hereby certifies as follows:

FIRST: The original Certificate of Incorporation of the Corporation was filed with the Secretary of State of the State of Delaware on March 7, 1996 under the name Aviron Merger Corporation. The Corporation filed an Amended and Restated Certificate of Incorporation on July 16, 1996; an Amended and Restated Certificate of Incorporation on November 22, 1996; and a Certificate of Amendment of the Amended and Restated Certificate of Incorporation on July 10, 2000.

SECOND: The Amended and Restated Certificate of Incorporation has been duly adopted in accordance with Sections 242 and 245 of the General Corporation Law of the State of Delaware by the director and the stockholder of the Corporation.

THIRD: The Certificate of Incorporation, as amended and restated, is hereby amended and restated to read in its entirety as follows.

ARTICLE I

The name of the Corporation is: Aviron

ARTICLE II

The address of the registered office of the Corporation in the State of Delaware is The Corporation Trust Company, Corporation Trust Center, 1209 Orange Street, in the City of Wilmington, County of New Castle 19801. The name of the Corporation's registered agent at such address is The Corporation Trust Company.

ARTICLE III

The purpose for which the Corporation is organized is to engage in any lawful acts or activities for which corporations may be organized under the General Corporation Law of the State of Delaware.

ARTICLE IV

The total number of shares of stock which the Corporation shall have authority to issue is one hundred shares of common stock, par value $.01, per share.

ARTICLE V

Elections of directors need not be by ballot unless required by the by-laws of the Corporation. Any director may be removed from office either with or without cause at any time by the affirmative vote of the holders of a majority of the outstanding stock of the Corporation entitled to vote, given at a meeting of the stockholders called for that purpose, or by the consent of the holders of a majority of the outstanding stock of the Corporation entitled to vote, given in accordance with Section 228 of the General Corporation Law of the State of Delaware.

ARTICLE VI

In furtherance and not in limitation of the power conferred upon the Board of Directors by law, the Board of Directors shall have power to make, adopt, alter, amend and repeal from time to time the by-laws of the Corporation, subject to the right of the stockholders entitled to vote with respect thereto to alter, amend and repeal by-laws adopted by the Board of Directors.

ARTICLE VII

No director shall be liable to the Corporation or any of its stockholders for monetary damages for breach of fiduciary duty as a director, provided that the foregoing shall not eliminate or limit any liability that may exist with respect to (1) a breach of the director's duty of loyalty to the Corporation or its stockholders, (2) acts or omissions not in good faith or which involve intentional misconduct or a knowing violation of law, (3) liability under Section 174 of the Delaware General Corporation Law or (4) a transaction from which the director derived an improper personal benefit, it being the intention of the foregoing provision to eliminate the liability of the Corporation's directors to the Corporation or its stockholders to the fullest extent permitted by Section 102(b)(7) of the Delaware General Corporation Law, as in effect on the date hereof and as such Section may be amended after the date hereof to the extent such amendment permits such liability to be further eliminated or limited. The Corporation shall indemnify to the fullest extent permitted by Section 145 of the Delaware General Corporation Law (as in effect on the date hereof and as such Section may be amended after the date hereof) each person that such Section grants the Corporation the power to indemnify.

2

IN WITNESS WHEREOF, Aviron has caused this certificate to be executed by its authorized officer, on this 15 day of January, 2002.

AVIRON

By: Charlene Friedman

Name: Charlene A. Friedman
Title: Vice President, General Counsel
and Secretary

Delaware

The First State

I, HARRIET SMITH WINDSOR, SECRETARY OF STATE OF THE STATE OF DELAWARE, DO HEREBY CERTIFY THE ATTACHED IS A TRUE AND CORRECT COPY OF THE CERTIFICATE OF AMENDMENT OF "AVIRON", CHANGING ITS NAME FROM "AVIRON" TO "MEDIMMUNE VACCINES, INC.", FILED IN THIS OFFICE ON THE TENTH DAY OF APRIL, A.D. 2002, AT 11 O'CLOCK A.M.

A FILED COPY OF THIS CERTIFICATE HAS BEEN FORWARDED TO THE NEW CASTLE COUNTY RECORDER OF DEEDS.

Harriet Smith Windsor

Harriet Smith Windsor, Secretary of State

2600915 8100

020228733

AUTHENTICATION: 1712941

DATE: 04-10-02

STATE OF DELAWARE
SECRETARY OF STATE
DIVISION OF CORPORATIONS
FILED 11:00 AM 04/10/2002
020228732 – 2600915

AVIRON

CERTIFICATE OF AMENDMENT TO AMENDED AND RESTATED CERTIFICATE OF INCORPORATION

AVIRON, a corporation organized and existing under the laws of the State of Delaware (the "Corporation"), hereby certifies as follows:

1. The Board of Directors of the Corporation, acting by the Written Consent of its Sole Director, duly adopted, pursuant to Section 242 of the General Corporation Law of the State of Delaware (the "GCL"), resolutions setting forth this proposed Amendment to the Amended and Restated Certificate. of Incorporation of said Corporation and declaring said Amendment to be advisable and directing that such Amendment be presented to the sole stockholder of the Corporation for consideration and approval;

2. The stockholder of the Corporation, acting by the Written Consent of its Sole Stockholder, approved and adopted this proposed Amendment to the Restated Certificate of Incorporation of said Corporation in accordance with Section 242 of the GCL;

3. Article 1 of the Amended and Restated Certificate of Incorporation of the Corporation, dated January 15, 2002, is hereby amended to read in full as follows:

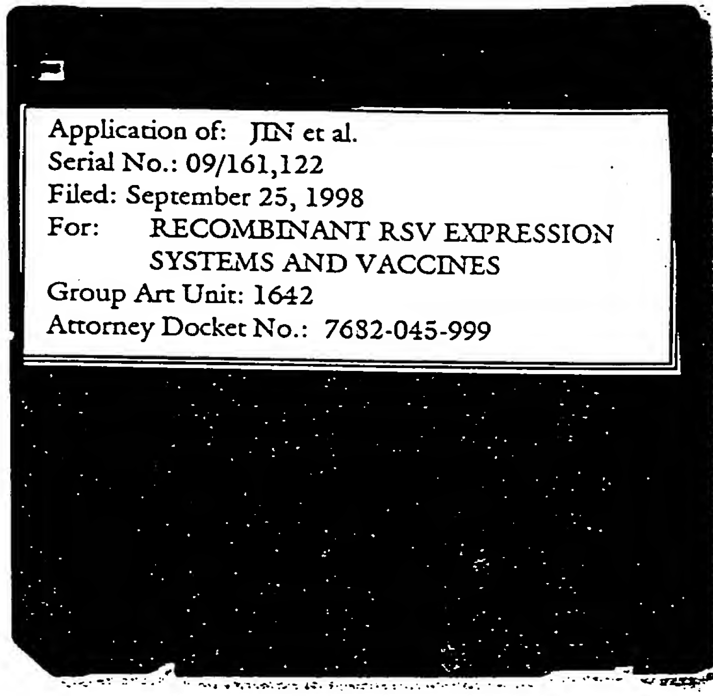
"The name of the Corporation is: MedImmune Vaccines, Inc."

IN WITNESS WHEREOF, AVIRON has caused this Certificate to be signed by David M. Mott, Chief Executive Officer, this day __ of April 2002.

AVIRON

By: ______________________
David M. Mott
Chief Executive Officer

NY—409404.3



Floppy Disk/CD Mailer

Application of: JIN et al.
Serial No.: 09/161,122
Filed: September 25, 1998
For: RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES

Group Art Unit: 1642
Examiner: Brumback, B.
Attorney Docket No.: 7682-045-999

SEQUENCE LISTING

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<120> Recombinant RSV Expression Systems and
Vaccines

<130> 7682-045

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1955 1960 1965

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Express Mail No.: EL 500 575 856 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: JIN et al.

Application No.: 09/161,122

Filed: September 25, 1998

For: RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES

Group Art Unit: 1642

Examiner: Brumback, B.

Attorney Docket No.: 7682-045-999

TRANSMITTAL OF FORMAL DRAWINGS

Assistant Commissioner for Patents
United States Patent and Trademark Office
(BOX PCT)
Washington, D.C. 20231

SIR/MADAM:

Applicants submit herewith 12 sheets of 12 formal figures to be substituted for the informal figures previously submitted in the above-identified application.

Respectfully submitted, by Jacqueline Ben
Reg No. 43,442

Date September 5, 2002

Laura A. Coruzzi 30,742

Laura A. Coruzzi (Reg. No.)

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New York, N.Y. 10036-2711
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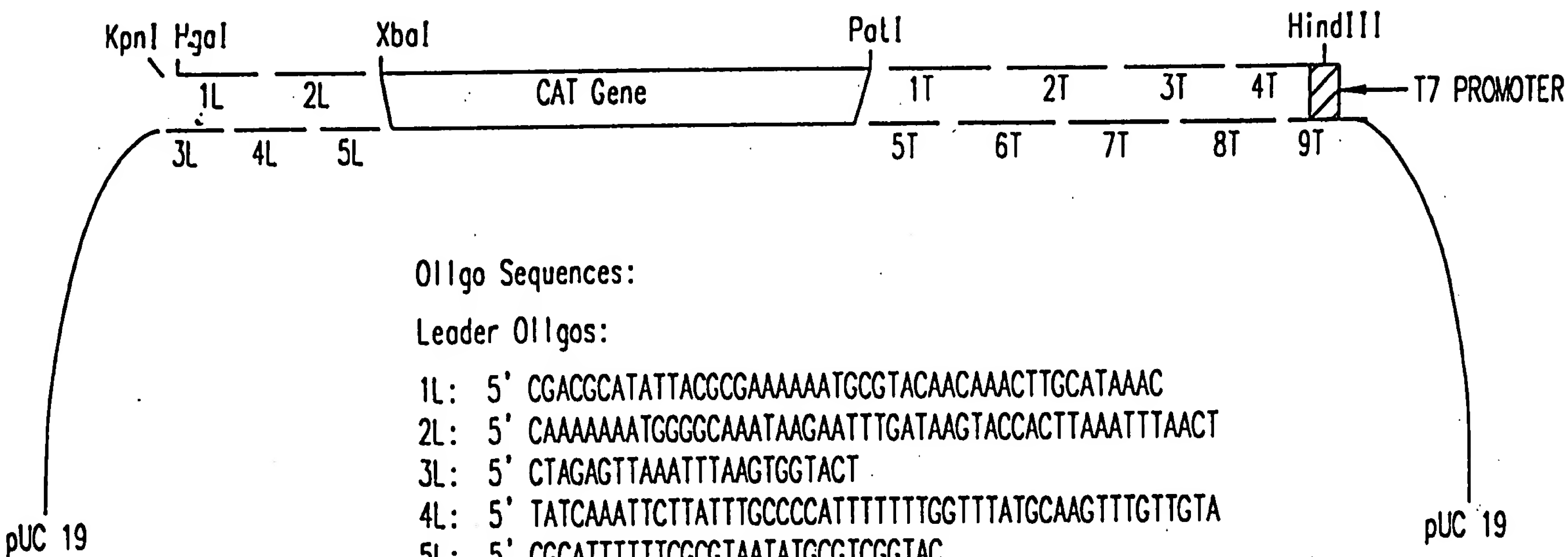


Oligo Sequences:

Leader Oligos:

1L: 5' CGACGCATATTACGCGAAAAAATGCGTACAACAAACTTGCATAAAC
2L: 5' CAAAAAAATGGGGCAAATAAGAATTTGATAAGTACCACTTAAATTTAACT
3L: 5' CTAGAGTTAAATTTAAGTGGTACT
4L: 5' TATCAAATTCTTATTTGCCCCATTTTTTTGGTTTATGCAAGTTTGTTGTA
5L: 5' CGCATTTTTTCGCGTAATATGCGTCGGTAC

Trailer Oligos:

1T: 5' GTATTCAATTATAGTTATTAAAAATTAAAAATCATATAATTTTTTAAATA
2T: 5' ACTTTTAGTGAACTAATCCTAAAGTTATCATTTTAATCTTGGAGGAATAA
3T: 5' ATTTAAACCCTAATCTAATTGGTTTATATGTGTATTAACTAAATTACGAG
4T: 5' ATATTAGTTTTTGACACTTTTTTTCTCGTTATAGTGAGTCGTATTA
5T: 5' AGCTTAATACGACTCACTATAACGA
6T: 5' GAAAAAAAGTGTCAAAAACTAATATCTCGTAATTTAGTTAATACACATAT
7T: 5' AAACCAATTAGATTAGGGTTTAAATTTATTCCTCCAAGATTAAAATGATA
8T: 5' ACTTTAGGATTAGTTCACTAAAAGTTATTTAAAAATTATATGATTTTTA
9T: 5' AATTTTAATAACTATAATTGAATACTGCA

FIG.1

Docket No.: 7682-045-999
Serial No.: 09/161,122
Inventor(s): Jin et al.
Title:Recombinant RSV Expression
Systems and Vaccines

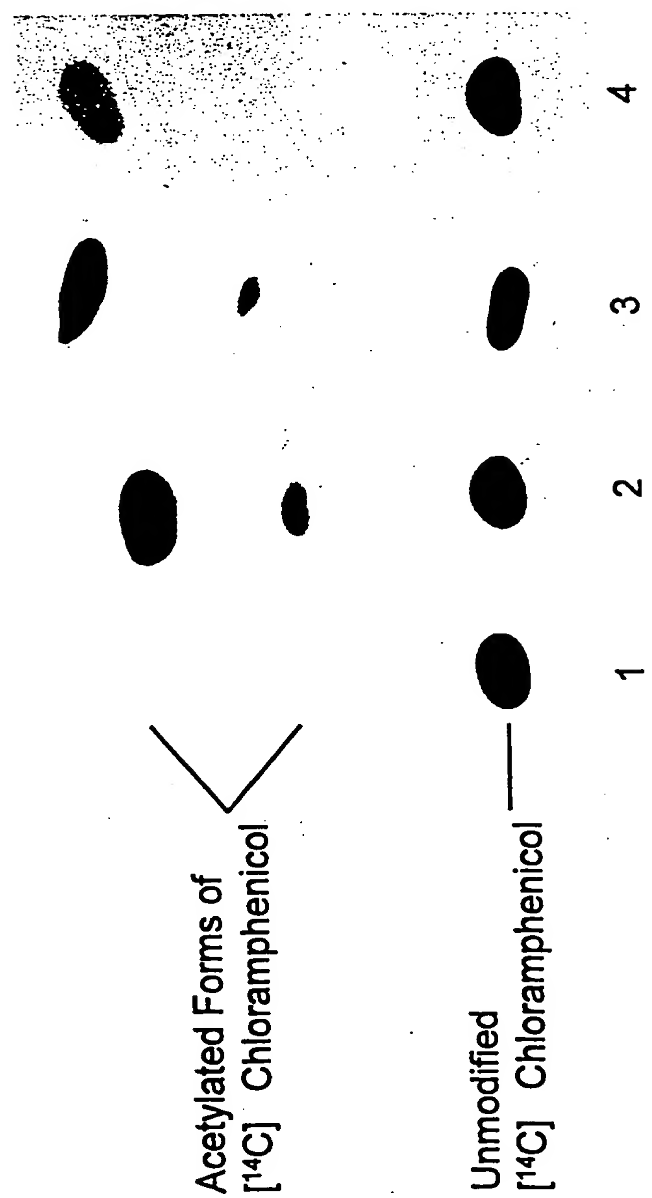
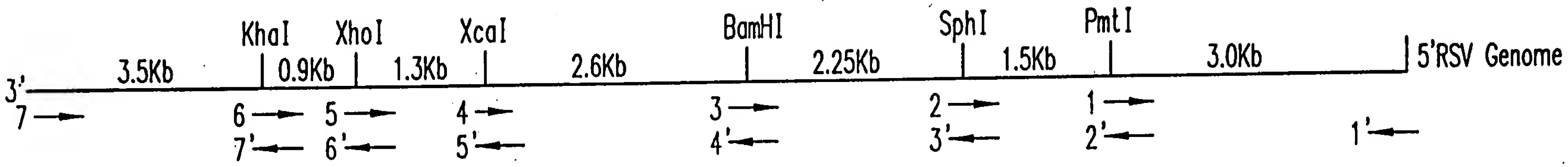


FIG.2



Primer Sequences:

1: 5' GTTTAACACGTGGTGAG
2: 5' ACATATAGGCATGCACC
3: 5' GACAAAATGGATCCCATT
4: 5' TGGTTGGTATACCAGTGT
5: 5' TACCAAGAGCTCGAGTCA
6: 5' TTTACCATATGCGCTAATGT
7: 5' ACGCGAAAAAAATGCGTACA

1: 5' ACGAGAAAAAAAGTGTCAA
2: 5' CTCACCACGTGTTAAAC
3: 5' GGTGCATGCCTATATGT
4: 5' AATGGGATCCATTTTGTCC
5: 5' AACACTGGTATACCAACCA
6: 5' TGACTCGAGCTCTTGGTA
7: 5' ACATTAGCGCATATGGTAAA

FIG.3

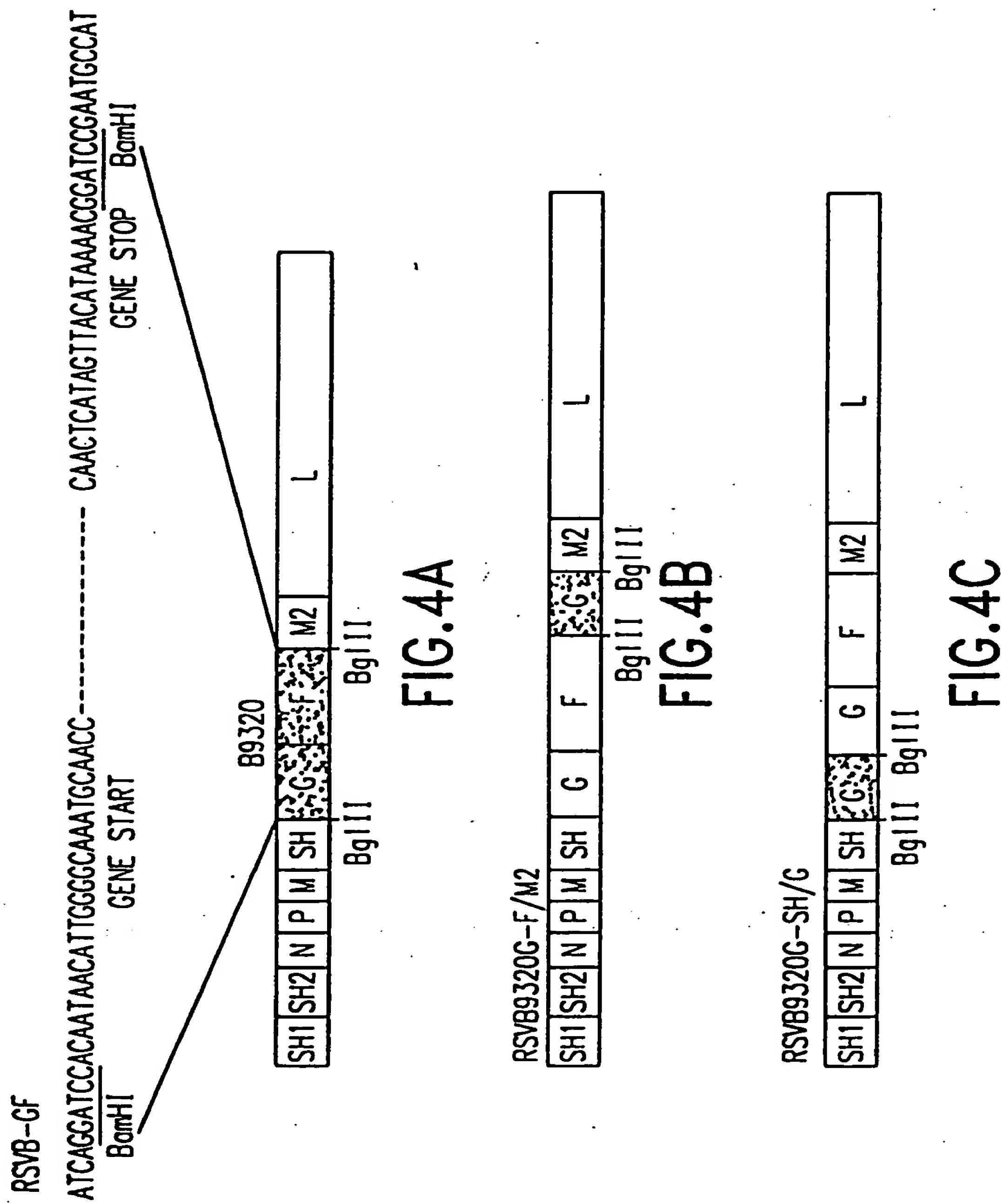


FIG.4A

FIG.4B

FIG.4C

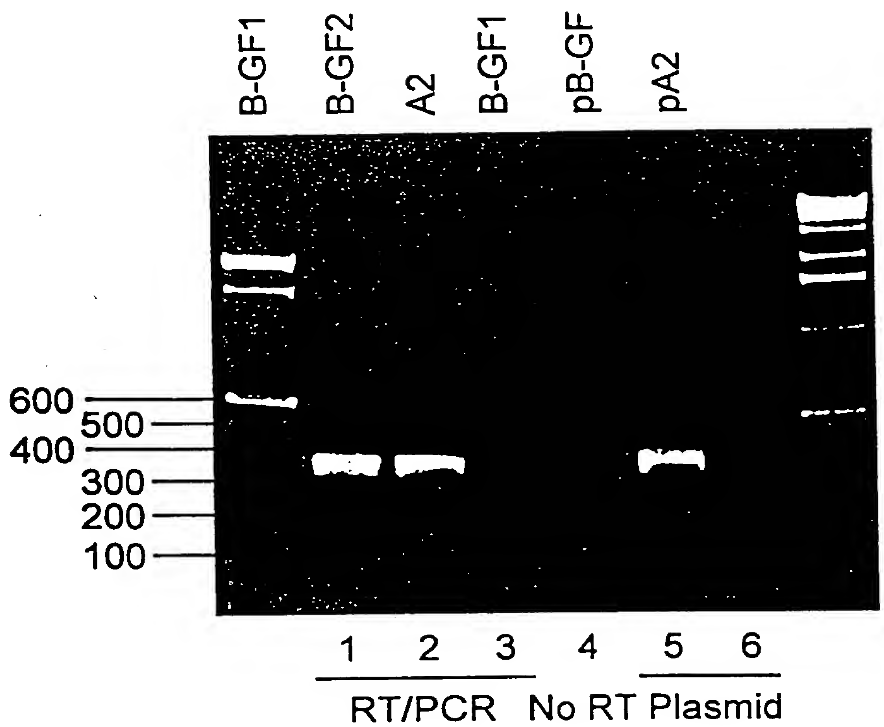

B-GF1
B-GF2
A2
B-GF1
pB-GF
pA2
600
500
400
300
200
100
1
2
3
4
5
6
RT/PCR
No RT
Plasmid

FIG.5

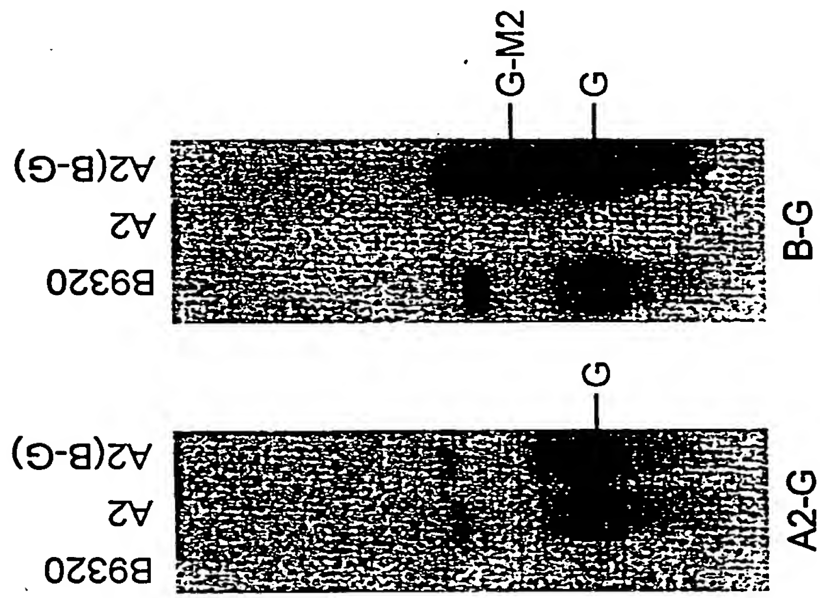

B9320
A2
A2(B-G)
G
A2-G
B9320
A2
A2(B-G)
G-M2
G
B-G

FIG.6B

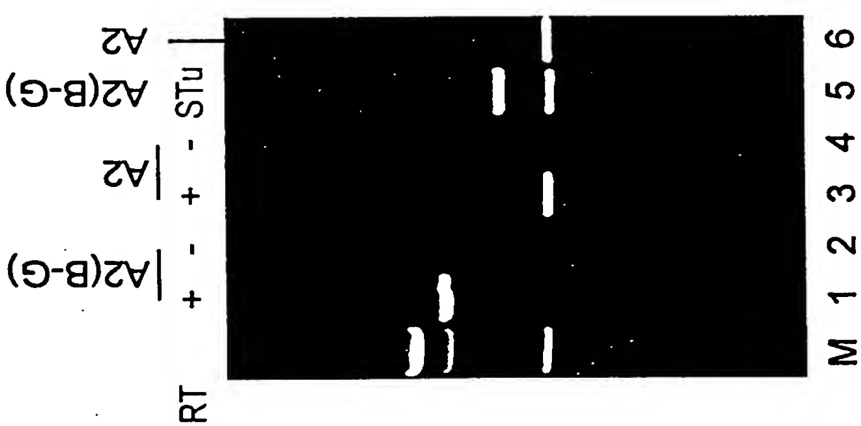

RT
+
-
A2(B-G)
+
-
A2
STu
A2(B-G)
A2
M
1
2
3
4
5
6

FIG.6A

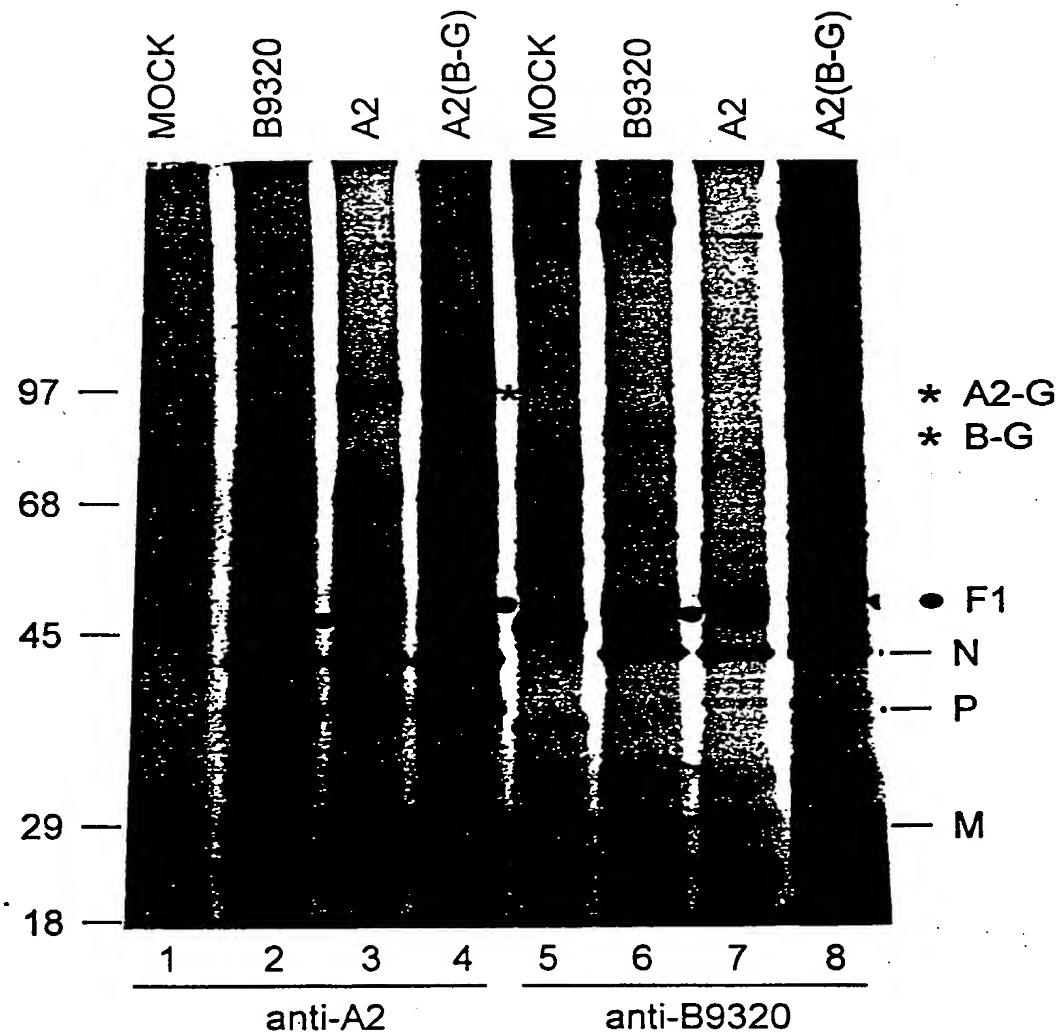

MOCK
B9320
A2
A2(B-G)
MOCK
B9320
A2
A2(B-G)
97
68
45
29
18
* A2-G
* B-G
● F1
N
P
M
1
2
3
4
5
6
7
8
anti-A2
anti-B9320

FIG.7

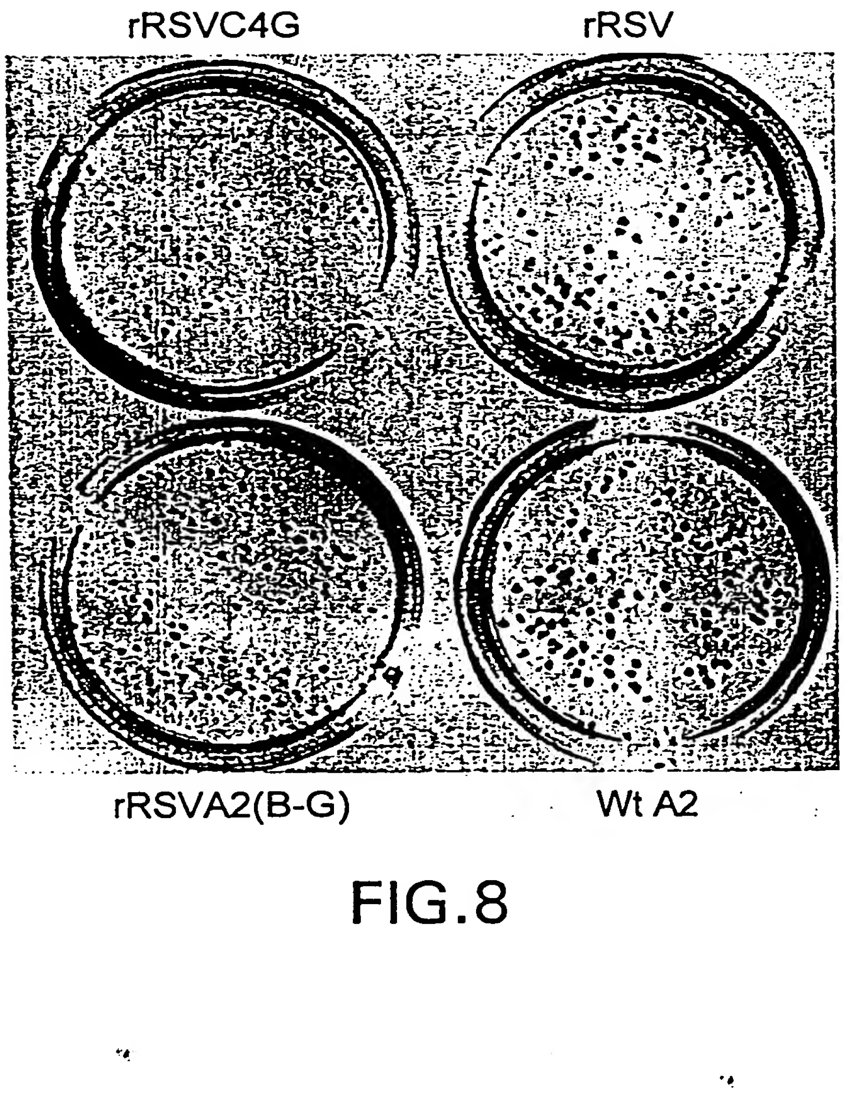


FIG.8

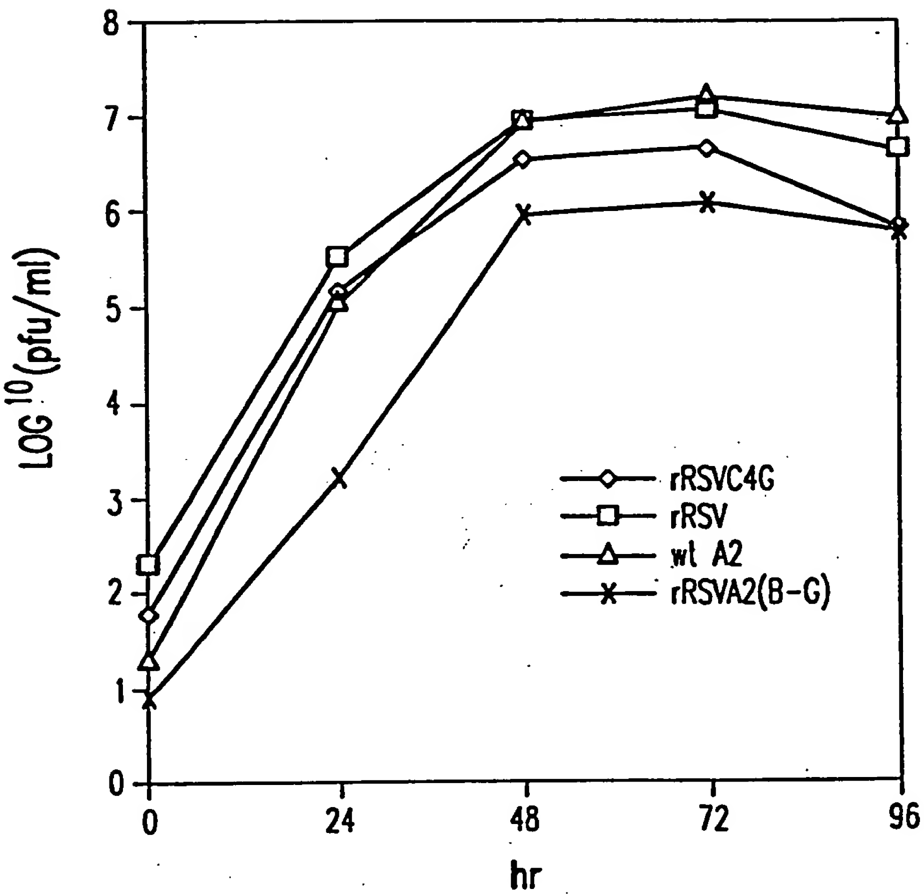

8
7
6
5
4
3
2
1
0
LOG 10(pfu/ml)
0
24
48
72
96
hr
rRSVC4G
rRSV
wt A2
rRSVA2(B-G)

FIG.9

```
MDPIINGNSANVYLT DSYLKGVISFSECNA LGSYIFNGPYLKNDY TNLISRQNPLIEHMN LKKLNITQSLISKYH 75
KGEIKLEEPTYFQSL LMTYKSMTSSEQIAT TNLLKKIIRRAIEIS DVKVYAILNKLGLKE KDKIKSNNGQDEDNS 150
VITTIIKDDILSAVK DNQSHLKADKNHSTK QRDTIKTTLLKKLMC SMQHPPSWLIHWFNL YTKLNNILTQYRSNE 225
VKNHGFTLIDNQTLS GFQFILNQYGCIVYH KELKRITVTTYNQFL TWKDISLSRLNVCLI TWISNCLNTLNKSLG 300
LRCGFNNVILTQLFL YGDCILKLFHNEGFY IIKEVEGFIMSLILN ITEEDQFRKREYNSM LNNITDAANKAQKNL 375
LSRVCHTLLDKTVSD NIINGRWIILLSKFL KLIKLAGDNNLNNLS ELYFLFRIFGHPMVD ERQAMDAVKINCNET 450
KFYLLSSLSMLRGAF IYRIIKGFVNNYNRW PTLRNAIVLPLRWLT YYKLNTYPSLLELTE RDLIVLSGLRFYREF 525
RLPKKVDLEMIINDK AISPPKNLIWTSFPR NYMPSHIQNYIEHEK LKFSESDKSRRVLEY YLRDNKFNECDLYNC 600
VVNQSYLNNPNHVVS LTGKERELSVGRMFA MQPGMFRQVQILAEK MIAENILQFFPESLT RYGDLELQKILELKA 675
GISNKSNRYNDNYNN YISKCSIITDLSKFN QAFRYETSCICSDVL DELHGVQSLFSWLHL TIPHVTIICTYRHAP 750
PYIGDHIVDLNNVDE QSGLYRYHMGGIEGW CQKLWTIEAISLLDL ISLKGKFSITALING DNQSIDISKPIRLME 825
GQTHAQADYLLALNS LKLLYKEYAGIGHKL KGTETYISRDMQFMS KTIQHNGVYYPASIK KVLPVGPWINTILDD 900
FKVSLESIGSLTQEL EYRGESLLCSLIFRN VWLYNQIALQLKNHA LCNNKLYLDILKVLK HLKTFFNLDNIDTAL 975
TLYMNLPMLFGGGDP NLLYRSFYRRTPDFL TEAIVHSVFILSYYT NHDLKDKLQDLSDDR LNKFLTCIITFDKNP 1050
NAEFVTLMRDPQALG SERQAKITSEINRLA VTEVLSTAPNKIFSK SAQHYTTTEIDLNDI MQNIEPTYPHGLRVV 1125
YESLPFYKAEKIVNL ISGTKSITNILEKTS AIDLTDIDRATEMMR KNITLLIRILPLDCN RDKREILSMENLSIT 1200
ELSKYVRERSWSLSN IVGVTSPSIMYTMDI KYTTSTISSGIIIEK YNVNSLTRGERGPTK PWVGSSTQEKKTMPV 1275
YNRQVLTKKQRDQID LLAKLDWVYASIDNK DEFMEELSIGTLGLT YEKAKKLFPQYLSVN YLHRLTVSSRPCEFP 1350
ASIPAYRTTNYHFDT SPINRILTEKYGDED IDIVFQNCISFGLSL MSVVEQFTNVCPNRI ILIPKLNEIHLMKPP 1425
IFTGDVDIHKLKQVI QKQHMFLPDKISLTQ YVELFLSNKTLKSGS HVNSNLILAHKISDY FHNTYILSTNLAGHW 1500
ILIIQLMKDSKGIFE KDWGEGYITDHMFIN LKVFFNAYKTYLLCF HKGYGKAKLECDMNT SDLLCVLELIDSSYW 1575
KSMSKVFLEQKVIKY ILSQDASLHRVKGCH SFKLWFLKRLNVAEF TVCPWVVNIDYHPTH MKAILTYIDLVRMGL 1650
INIDRIHIKNKHKFN DEFYTSNLFYINYNF SDNTHLLTKHIRIAN SELENNYNKLYHPTP ETLENILANPIKSND 1725
KKTLNDYCIGKNVDS IMLPLLSNKKLIKSS AMIRTNYSKQDLYNL FPMVVIDRIIDHSGN TAKSNQLYTTTSHQI 1800
SLVHNSTSLYCMLPW HHINRFNFVFSSTGC KISIEYILKDLKIKD PNCIAFIGEGAGNLL LRTVVELHPDIRYIY 1875
RSLKDCNDHSLPIEF LRLYNGHINIDYGEN LTIPATDATNNIHWS YLHIKFAEPISLFVC DAELSVTVNWSKIII 1950
EWSKHVRKCKYCSSV NKCMLIVKYHAQDDI DFKLDNITILKTYVC LGSKLKGSEVYLVLT IGPANIFPVFNVVQN 2025
AKLILSRTKNFIMPK KADKESIDANIKSLI PFLCYPITKKGINTA LSKLKSVVSGDILSY SIAGRNEVFSNKLIN 2100
HKHMNILKWFNHVLN FRSTELNYNHLYMVE STYPYLSELLNSLTT NELKKLIKITGSLLY NFHNE           2165
```

Charged Clusters (Amino Acids that are underlined were changed to alanines)
Mutations in cpts-248/404
Mutation in cpts530

FIG.10

Docket No.: 7682-045-999
Serial No.: 09/161,122
Inventor(s): Jin et al.
Title:Recombinant RSV Expression
Systems and Vaccines

```
MDPIINGNSANVYLT DSYLKGVISFSECNA LGSYIFNGPYLKNDY TNLISRQNPLIEHMN LKKLNITQSLISKYH   75
KGEIKLEEPTYFQSL LMTYKSMTSSEQIAT TNLLKKIIRRAIEIS DVKVYAILNKLGLKE KDKIKSNNGQDEDNS  150
VITTIIKDDILSAVK DNQSHLKADKNHSTK QRDTIKTTLLKKLMC SMQHPPSWLIHWFNL YTKLNNILTQYRSNE  225
VKNHGFTLIDNQTLS GFQFILNQYGCIVYH KELKRITVTTYNQFL TWKDISLSRLNVCLI TWISNCLNTLNKSLG  300
LRCGFNNVILTQLFL YGDCILKLFHNEGFY IIKEVEGFIMSLILN ITEEDQFRKREYNSM LNNITDAANKAQKNL  375
LSRVCHTLLDKTVSD NIINGRWIILLSKFL KLIKLAGDNNLNNLS ELYFLFRIFGHPMVD ERQAMDAVKINCNET  450
KFYLLSSLSMLRGAF IYRIIKGFVNNYNRW PTLRNAIVLPLRWLT YYKLNTYPSLLELTE RDLIVLSGLRFYREF  525
RLPKKVDLEMIINDK AISPPKNLIWTSFPR NYMPSHIQNYIEHEK LKFSESDKSRRVLEY YLRDNKFNECDLYNC  600
VVNQSYLNNPNHVVS LTGKERELSVGRMFA MQPGMFRQVQILAEK MIAENILQFFPESLT RYGDLELQKILELKA  675
GISNKSNRYNDNYNN YISKCSIITDLSKFN QAFRYETSCICSDVL DELHGVQSLFSWLHL TIPHVTIICTYRHAP  750
PYIGDHIVDLNNVDE QSGLYRYHMGGIEGW CQKLWTIEAISLLDL ISLKGKFSITALING DNQSIDISKPIRLME  825
GQTHAQADYLLALNS LKLLYKEYAGIGHKL KGTETYISRDMQFMS KTIQHNGVYYPASIK KVLPVGPWINTILDD  900
FKVSLESIGSLTQEL EYRGESLLCSLIFRN VWLYNQIALQLKNHA LCNNKLYLDILKVLK HLKTFFNLDNIDTAL  975
TLYMNLPMLFGGGDP NLLYRSFYRRTPDFL TEAIVHSVFILSYYT NHDLKDKLQDLSDDR LNKFLTCIITFDKNP 1050
NAEFVTLMRDPQALG SERQAKITSEINRLA VTEVLSTAPNKIFSK SAQHYTTTEIDLNDI MQNIEPTYPHGLRVV 1125
YESLPFYKAEKIVNL ISGTKSITNILEKTS AIDLTDIDRATEMMR KNITLLIRILPLDCN RDKREILSMENLSIT 1200
ELSKYVRERSWSLSN IVGVTSPSIMYTMDI KYTTSTISSGIIIEK YNVNSLTRGERGPTK PWVGSSTQEKKTMPV 1275
YNRQVLTKKQRDQID LLAKLDWVYASIDNK DEFMEELSIGTLGLT YEKAKKLFPQYLSVN YLHRLTVSSRPCEFP 1350
ASIPAYRTTNYHFDT SPINRILTEKYGDED IDIVFQNCISFGLSL MSVVEQFTNVCPNRI ILIPKLNEIHLMKPP 1425
IFTGDVDIHKLKQVI QKQHMFLPDKISLTQ YVELFLSNKTLKSGS HVNSNLILAHKISDY FHNTYILSTNLAGHW 1500
ILIIQLMKDSKGIFE KDWGEGYITDHMFIN LKVFFNAYKTYLLCF HKGYGKAKLECDMNT SDLLCVLELIDSSYW 1575
KSMSKVFLEQKVIKY ILSQDASLHRVKGCH SFKLWFLKRLNVAEF TVCPWVVNIDYHPTH MKAILTYIDLVRMGL 1650
INIDRIHIKNKHKFN DEFYTSNLFYINYNF SDNTHLLTKHIRIAN SELENNYNKLYHPTP ETLENILANPIKSND 1725
KKTLNDYCIGKNVDS IMLPLLSNKKLIKSS AMIRTNYSKQDLYNL FPMVVIDRIIDHSGN TAKSNQLYTTTSHQI 1800
SLVHNSTSLYCMLPW HHINRFNFVFSSTGC KISIEYILKDLKIKD PNCIAFIGEGAGNLL LRTVVELHPDIRYIY 1875
RSLKDCNDHSLPIEF LRLYNGHINIDYGEN LTIPATDATNNIHWS YLHIKFAEPISLFVC DAELSVTVNWSKIII 1950
EWSKHVRKCKYCSSV NKCMLIVKYHAQDDI DFKLDNITILKTYVC LGSKLKGSEVYLVLT IGPANIFPVFNVVQN 2025
AKLILSRTKNFIMPK KADKESIDANIKSLI PFLCYPITKKGINTA LSKLKSVVSGDILSY SIAGRNEVFSNKLIN 2100
HKHMNILKWFNHVLN FRSTELNYNHLYMVE STYPYLSELLNSLTT NELKKLIKITGSLLY NFHNE           2165
```

C Cysteine residues
C Cysteine residues that were changed to valine or aspartic acid
C Cysteine residues deleted

FIG.11

Docket No.: 7682-045-999
Serial No.: 09/161,122
Inventor(s): Jin et al.
Title: Recombinant RSV Expression Systems and Vaccines

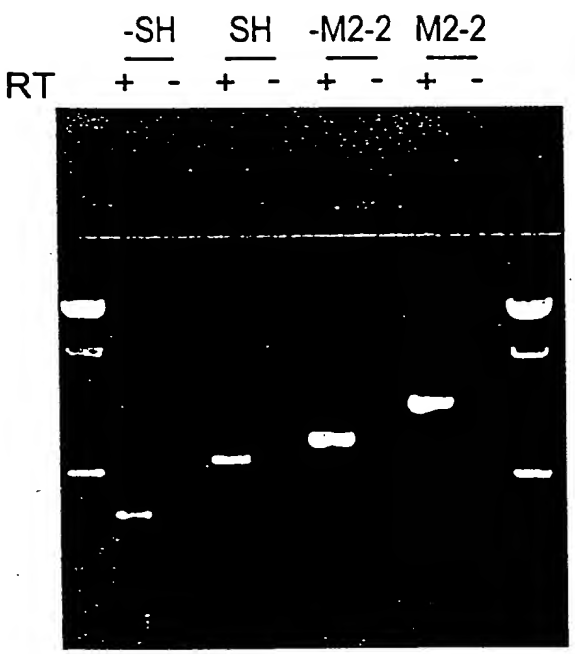


FIG.12A

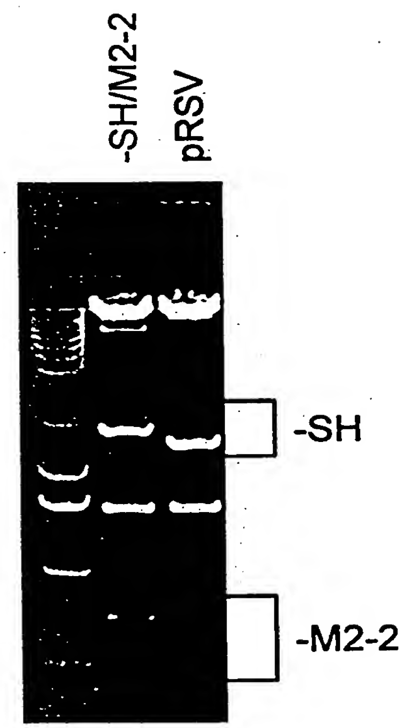


FIG.12B

US006033886A

United States Patent [19]

Conzelmann

[11] **Patent Number: 6,033,886**

[45] **Date of Patent: Mar. 7, 2000**

[54] **RECOMBINANT INFECTIOUS NON-SEGMENTED NEGATIVE STRAND RNA VIRUS**

[76] Inventor: **Karl Klaus Conzelmann**, Lilienstrasse 9, D-72406 Bisingen, Germany

[21] Appl. No.: **08/808,130**

[22] Filed: **Feb. 28, 1997**

Related U.S. Application Data

[63] Continuation of application No. 08/503,351, Jul. 18, 1995, abandoned.

[30] **Foreign Application Priority Data**

Jul. 18, 1994 [EP] European Pat. Off. 94202089

[51] **Int. Cl.**[7] C12N 15/00; A61K 39/12; A61K 39/155; A61K 39/165

[52] **U.S. Cl.** 435/172.3; 424/205.1; 424/211.1; 424/212.1; 424/224.1; 424/93.6; 435/235.1; 435/236; 435/172.1

[58] **Field of Search** 435/172.3, 235.1, 435/236, 172.1; 424/205.1, 211.1, 212.1, 224.1, 93.6

[56] **References Cited**

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WO-A-9408022 4/1994 WIPO .

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K.K. Conzelmann et al., "Rescue of Synthetic Genome Analogs of Rabies Virus by Plasmid Encoded Proteins," *J. Virol.*, 68:2:713–719, 1994.

W. Luytjes et al., "Amplification, Packaging and Expression of a Foreign gene by influenzy," *Cell* 59:6:1107–1113, 1989.

S. Li et al., "Chimeric Influenza Virus Induces Neutralising Antibodies," *J. Virol.*, 67:12:6659–6666, 1993.

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Crowe J, Bui P, London W, Davis A, Hung P, Chanock R, and Murphy B. Satisfactorily attenuated and protective mutants derived from a partially attenuated cold-passaged respiratory syncytial virus mutant by introduction of additional attenuating mutations. Vaccine 12(8): 691–699, 1994.

(List continued on next page.)

Primary Examiner—Jeffrey Stucker
Assistant Examiner—Hankyel T. Park
Attorney, Agent, or Firm—Michael G. Sullivan

[57] **ABSTRACT**

The present invention provides the generation of infectious replicating non-segmented negative-stranded RNA virus, entirely from cloned cDNA. This process offers the possibility to introduce mutations into the virus genome by means of recombinant DNA techniques.

19 Claims, 15 Drawing Sheets

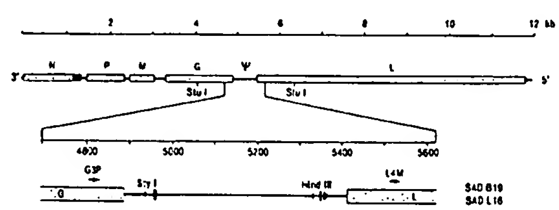

Exhibit E

US006033886A

United States Patent [19]

Conzelmann

[11] **Patent Number:** **6,033,886**

[45] **Date of Patent:** **Mar. 7, 2000**

[54] **RECOMBINANT INFECTIOUS NON-SEGMENTED NEGATIVE STRAND RNA VIRUS**

[76] Inventor: **Karl Klaus Conzelmann**, Lilienstrasse 9, D-72406 Bisingen, Germany

[21] Appl. No.: **08/808,130**

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[51] Int. Cl.^{7} **C12N 15/00**; A61K 39/12; A61K 39/155; A61K 39/165

[52] U.S. Cl. **435/172.3**; 424/205.1; 424/211.1; 424/212.1; 424/224.1; 424/93.6; 435/235.1; 435/236; 435/172.1

[58] Field of Search 435/172.3, 235.1, 435/236, 172.1; 424/205.1, 211.1, 212.1, 224.1, 93.6

[56] **References Cited**

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S. Li et al., "Chimeric Influenza Virus Induces Neutralising Antibodies," *J. Virol.*, 67:12:6659–6666, 1993.

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Crowe J, Bui P, London W, Davis A, Hung P, Chanock R, and Murphy B. Satisfactorily attenuated and protective mutants derived from a partially attenuated cold–passaged respiratory syncytial virus mutant by introduction of additional attenuating mutations. Vaccine 12(8): 691–699, 1994.

(List continued on next page.)

Primary Examiner—Jeffrey Stucker
Assistant Examiner—Hankyel T. Park
Attorney, Agent, or Firm—Michael G. Sullivan

[57] **ABSTRACT**

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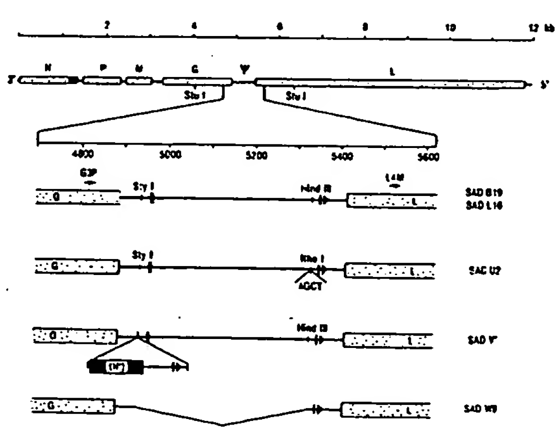
19 Claims, 15 Drawing Sheets



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Boyer J, Haenni A. Infectious transcripts and cDNA clones of RNA Viruses. Virology 198: 415–426, 1994.

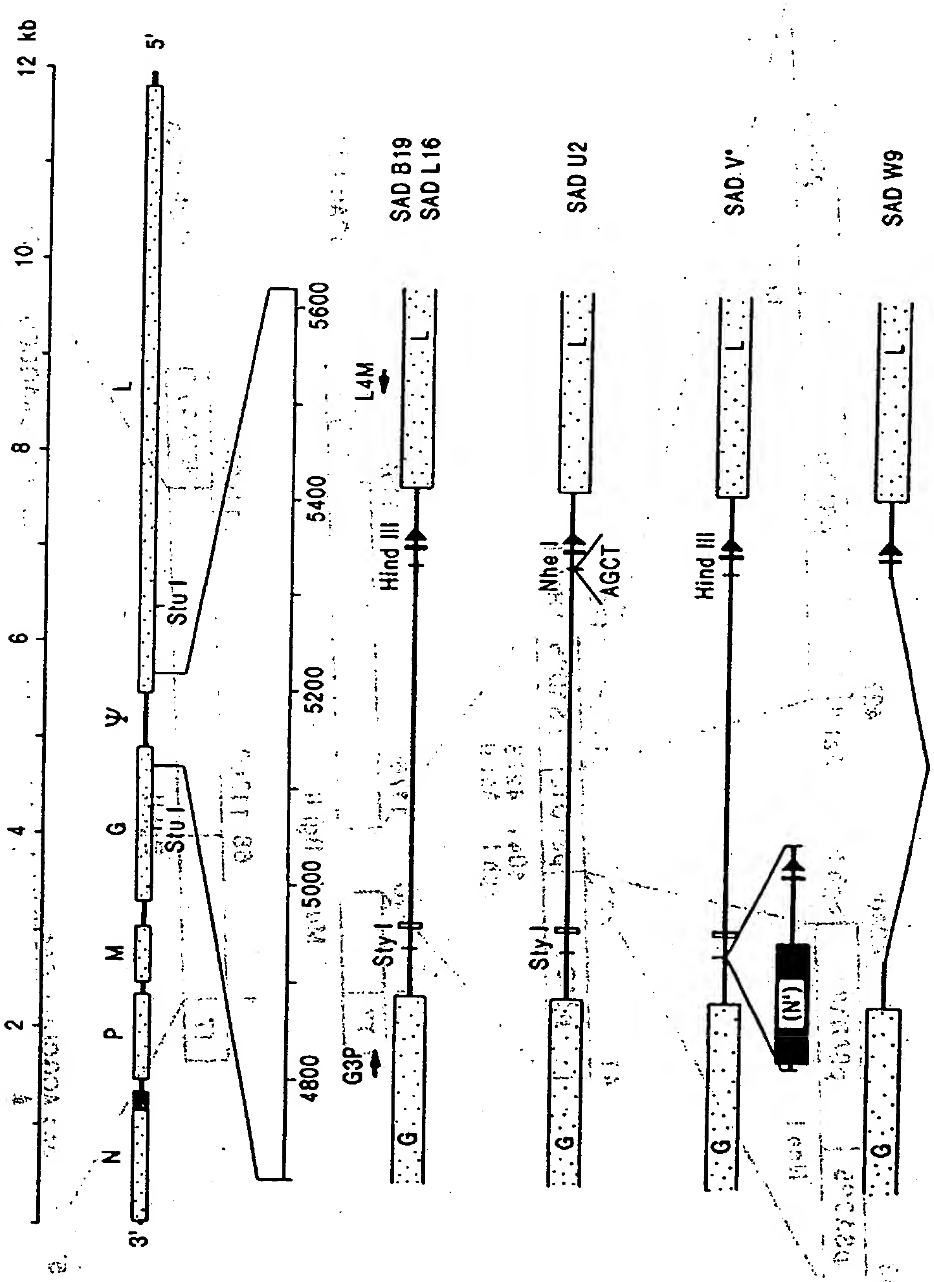
12 kb
10
8
6
4
2
5'
3'
N
P
M
G
Ψ
L
Stu I
Stu I
5600
5400
5200
5000
4800
SAD B19
SAD L16
SAD U2
SAD V*
SAD W9
L4M
G3P
Hind III
Sty I
Nhe I
AGCT
Sty I
Hind III
(N')

FIG. 1

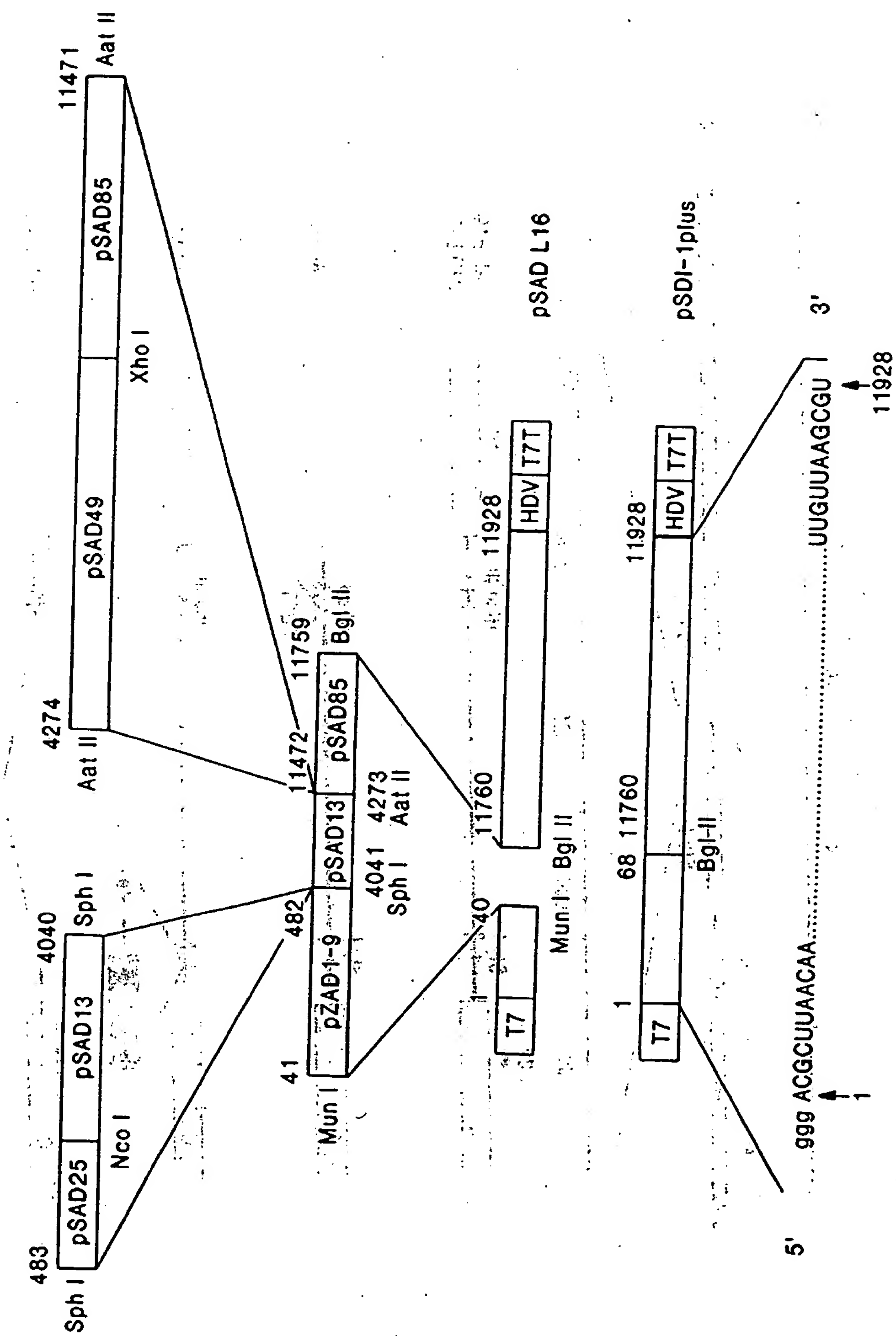
Sph I
483
pSAD25
Nco I
pSAD13
4040
Sph I
Aat II
4274
pSAD49
Xho I
pSAD85
11471
Aat II
Mun I
41
pZAD1-9
482
pSAD13
4041
Sph I
4273
Aat II
11472
pSAD85
11759
Bgl II
T7
40
Mun I
Bgl II
11760
11928
HDV
T7T
pSAD L16
1
T7
68
Bgl-II
11760
11928
HDV
T7T
pSDI-1plus
5'
ggg ACGCUUAACAA........UUGUUAAGCGU
1
11928
3'

FIG. 2

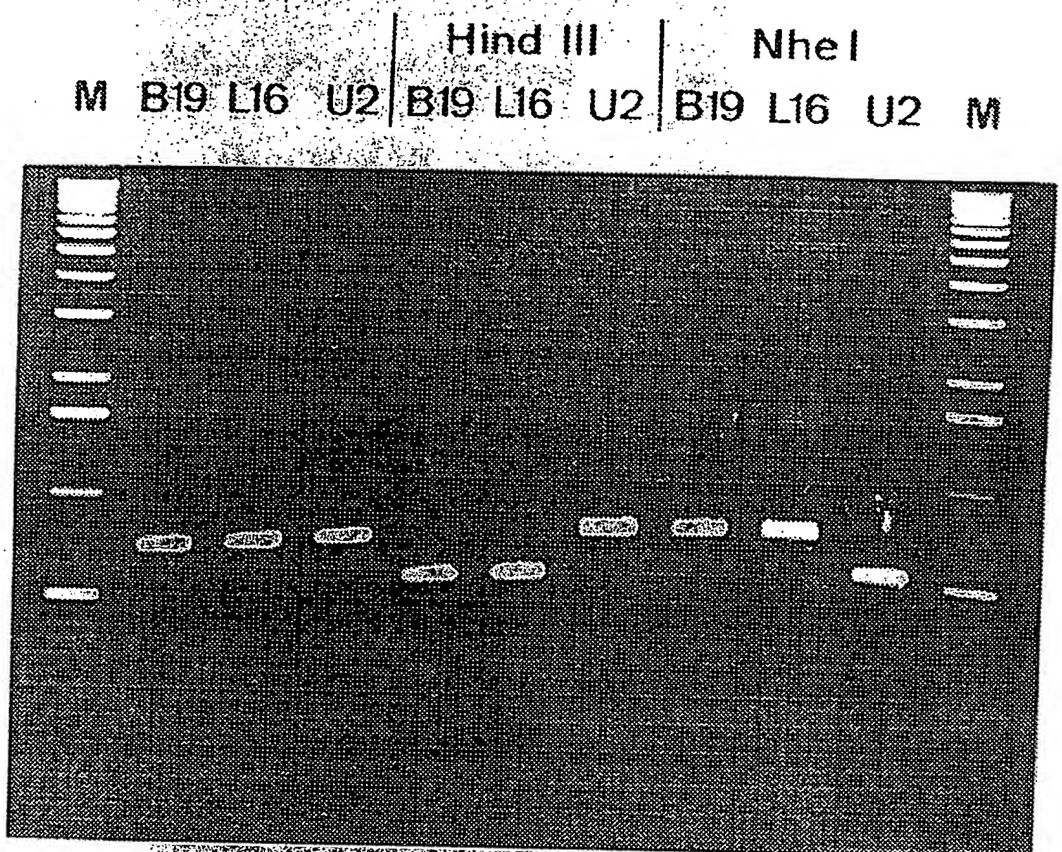

FIG. 3

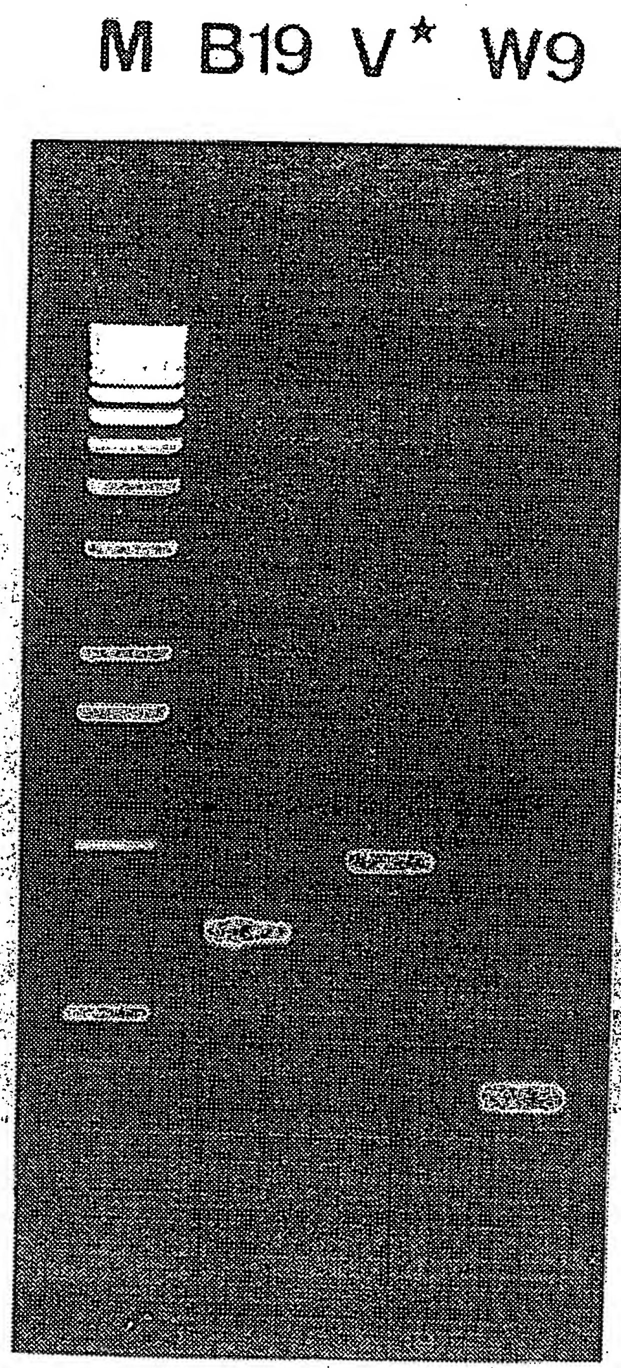

FIG. 4

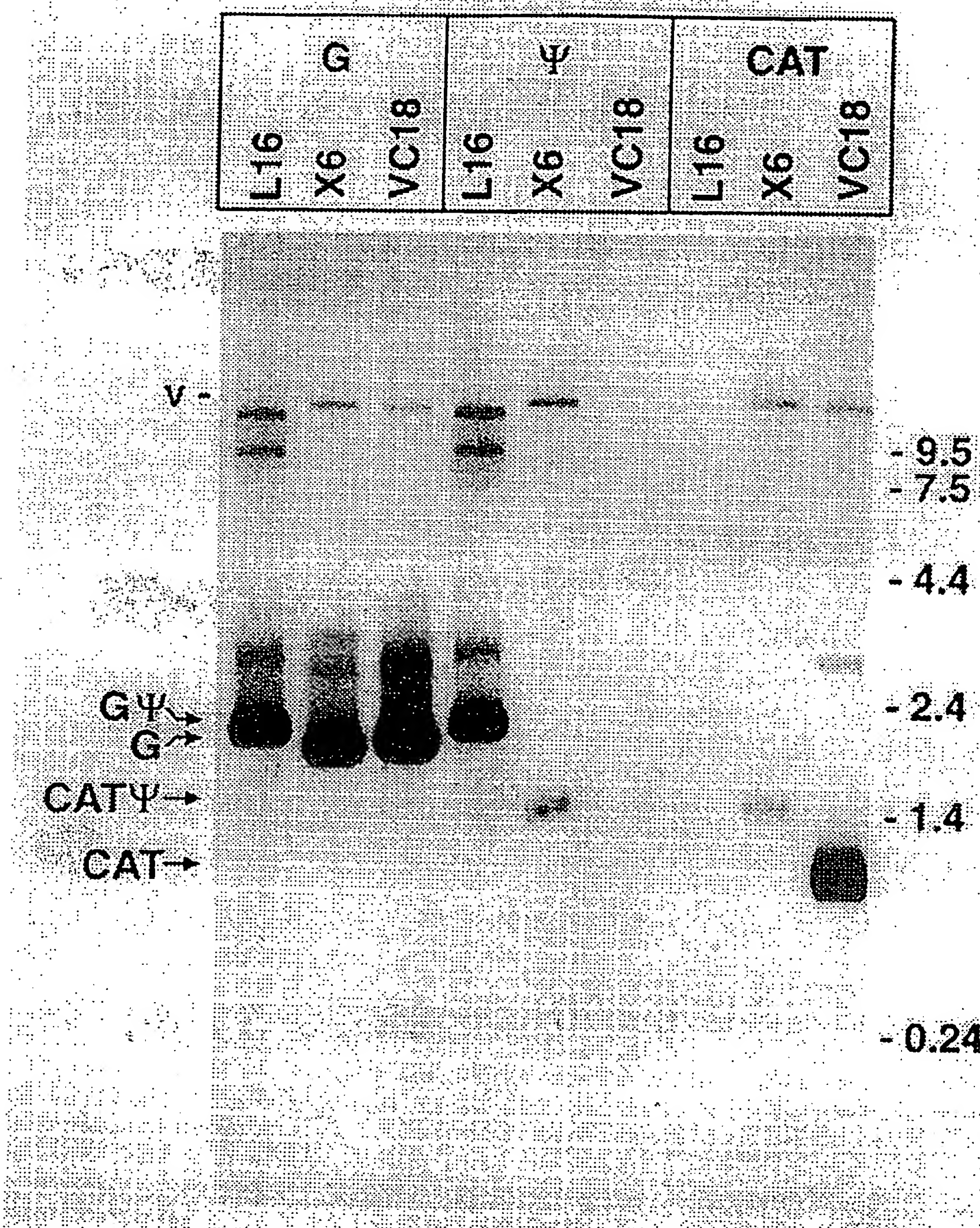
G
Ψ
CAT
L16
X6
VC18
L16
X6
VC18
L16
X6
VC18
V
GΨ
G
CATΨ
CAT
9.5
7.5
4.4
2.4
1.4
0.24

FIG. 5

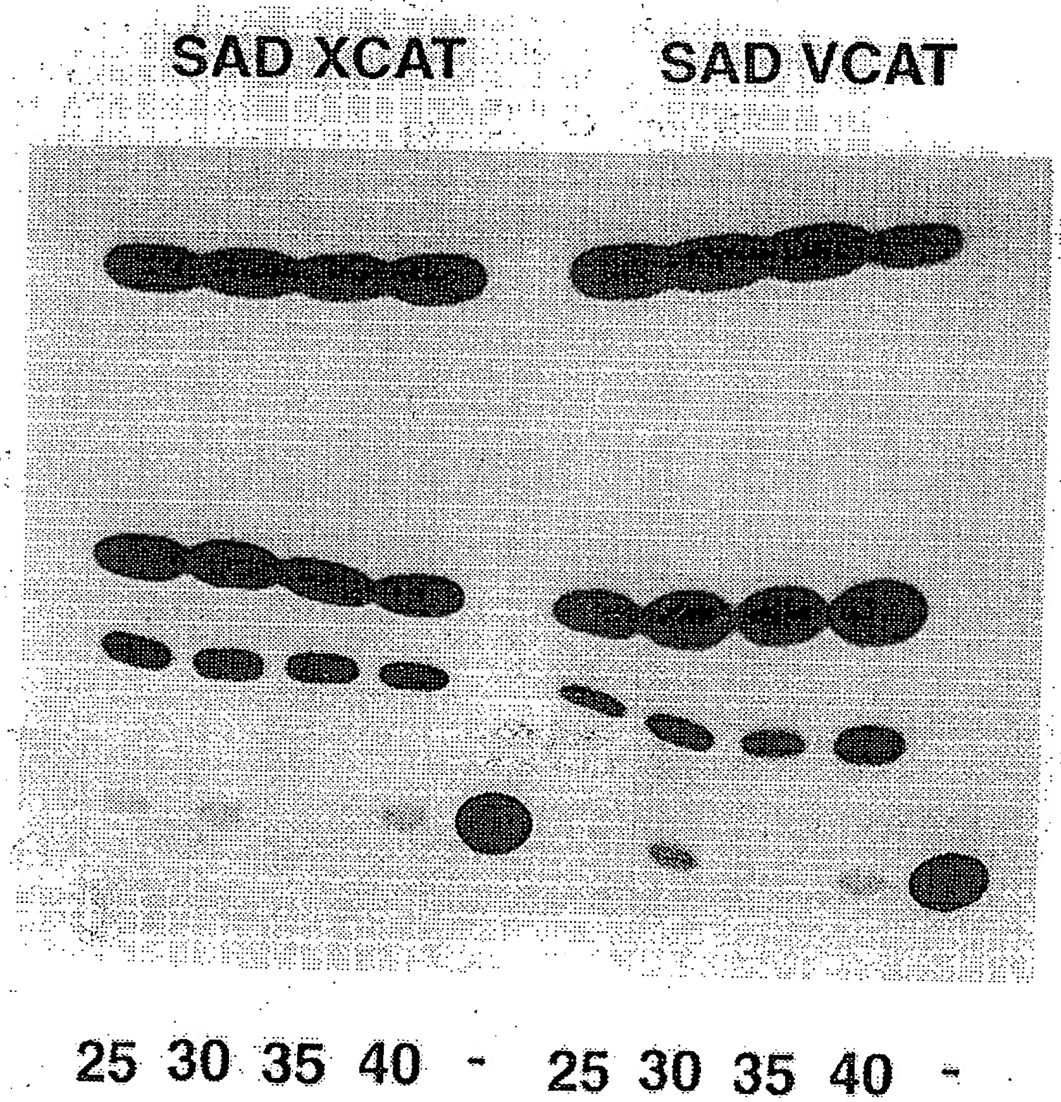

FIG. 6

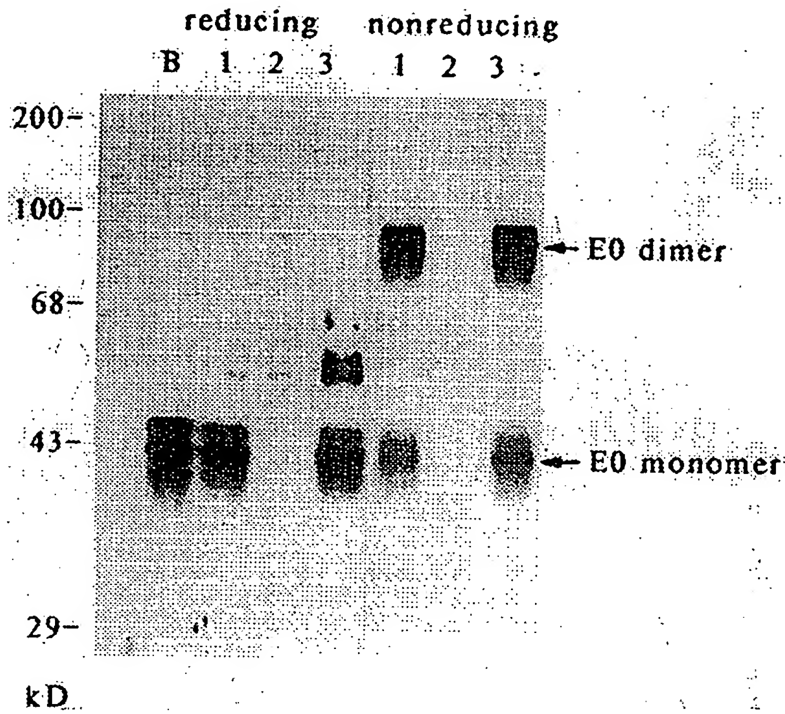
reducing
nonreducing
B
1
2
3
1
2
3
200-
100-
68-
43-
29-
kD
E0 dimer
E0 monomer

FIG. 7A

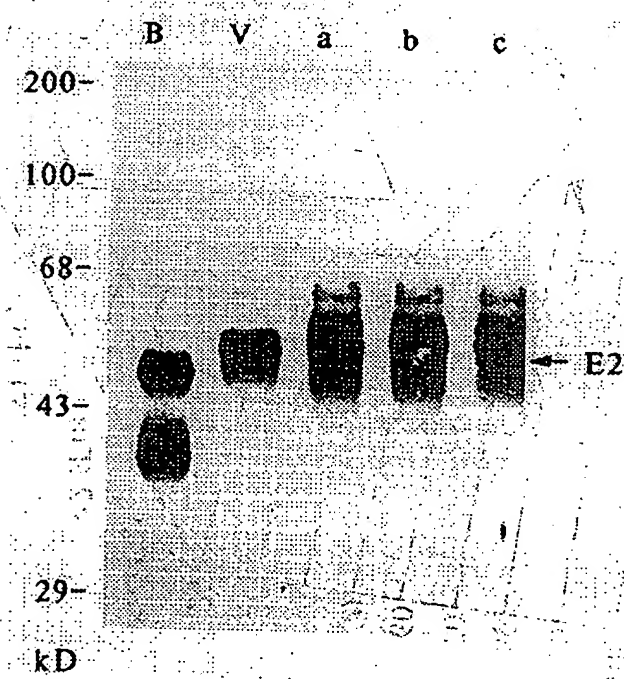
B
V
a
b
c
200-
100-
68-
43-
29-
kD
E2

FIG. 7B

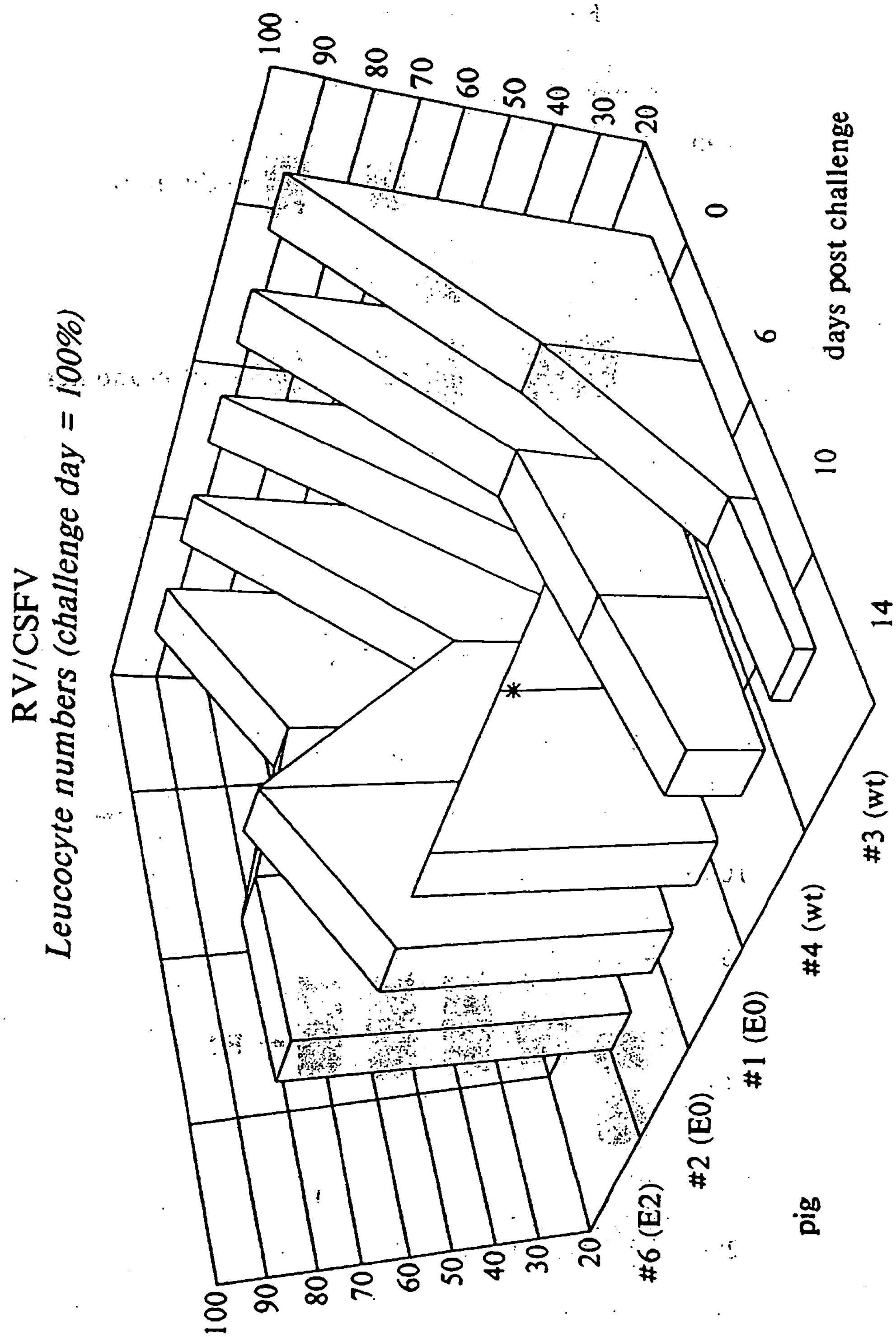
RV/CSFV
Leucocyte numbers (challenge day = 100%)
100
90
80
70
60
50
40
30
20
days post challenge
0
6
10
14
pig
#6 (E2)
#2 (E0)
#1 (E0)
#4 (wt)
#3 (wt)

FIG. 8

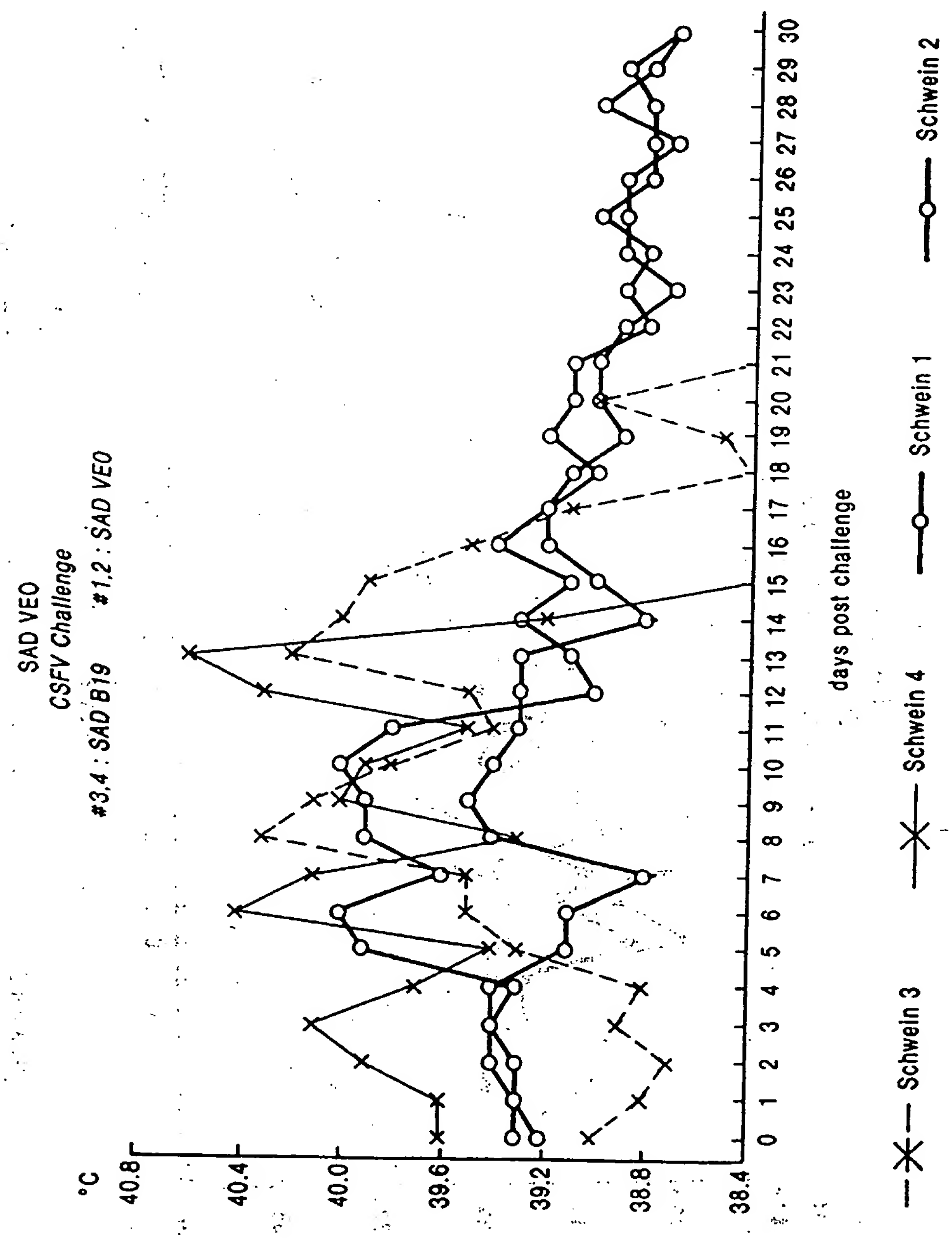
SAD VEO
CSFV Challenge
#3,4 : SAD B19 #1,2 : SAD VEO
°C
40.8
40.4
40.0
39.6
39.2
38.8
38.4
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
days post challenge
Schwein 3
Schwein 4
Schwein 1
Schwein 2

FIG. 9A

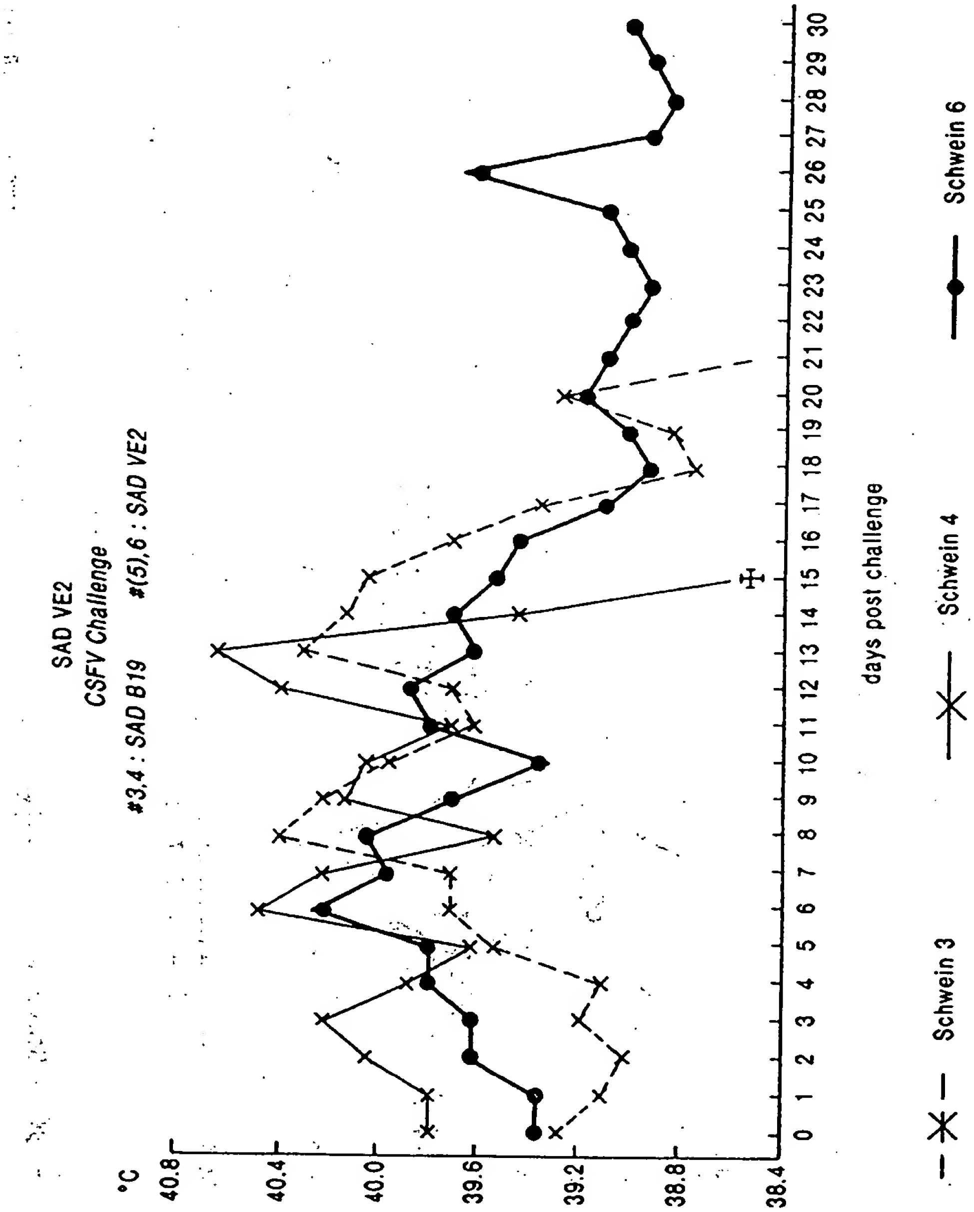
SAD VE2
CSFV Challenge
#3,4 : SAD B19 #(5),6 : SAD VE2
°C
40.8
40.4
40.0
39.6
39.2
38.8
38.4
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
days post challenge
Schwein 3
Schwein 4
Schwein 6

FIG. 9B

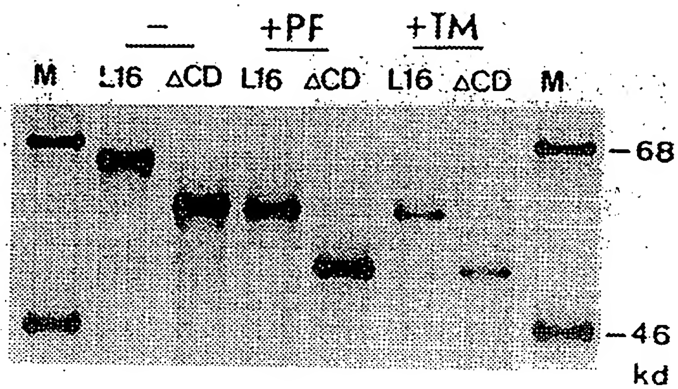

FIG. 10A

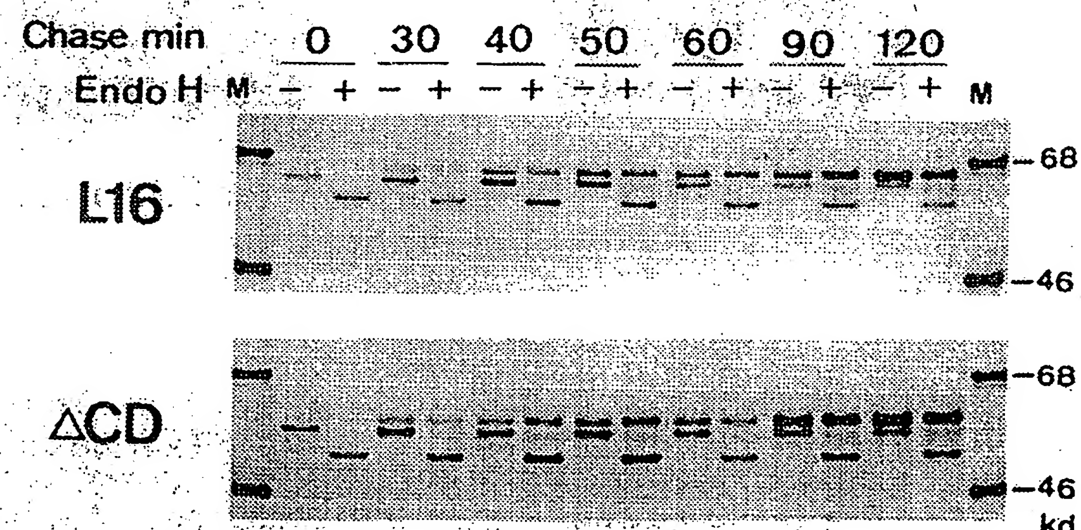

FIG. 10B

L16

24h

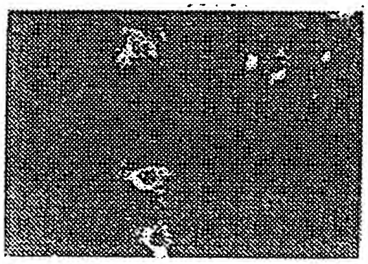

FIG. 11A

L16

36h

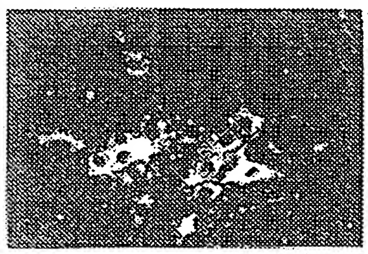

FIG. 11B

L16

48h

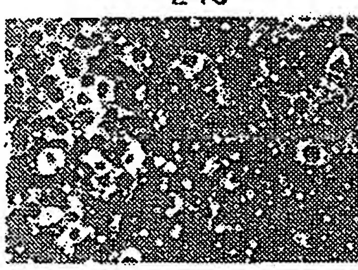

FIG. 11C

ΔCD

24h

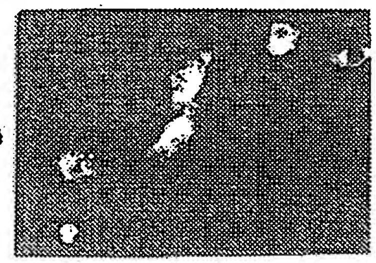

FIG. 11D

ΔCD

36h

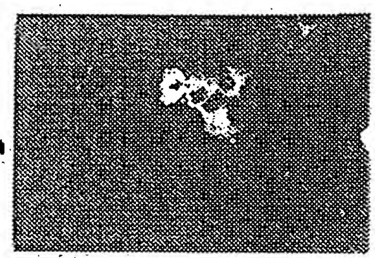

FIG. 11E

ΔCD

48h

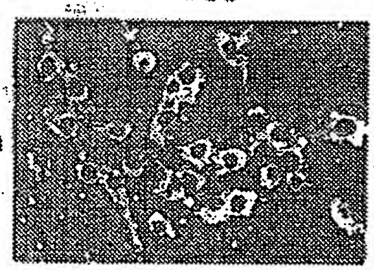

FIG. 11F

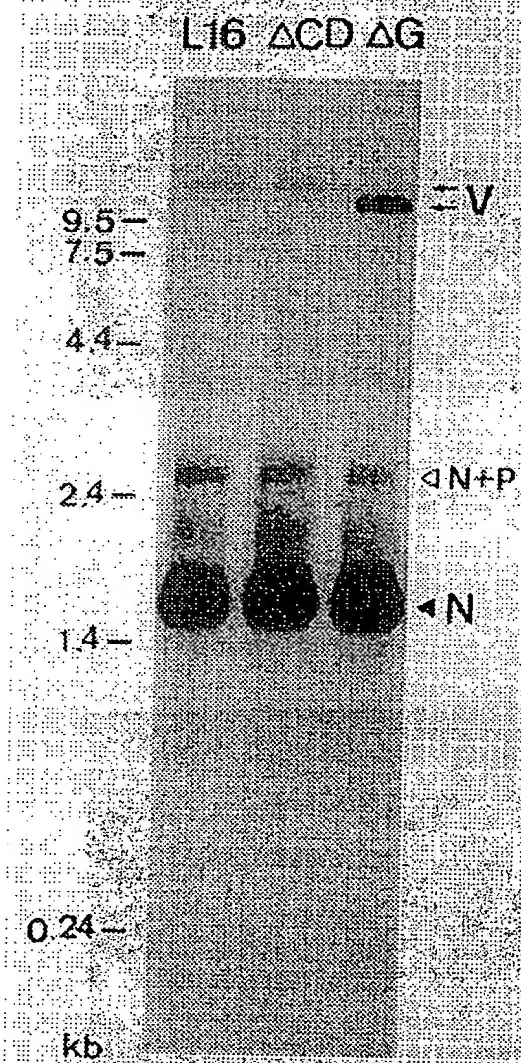

FIG. 12A

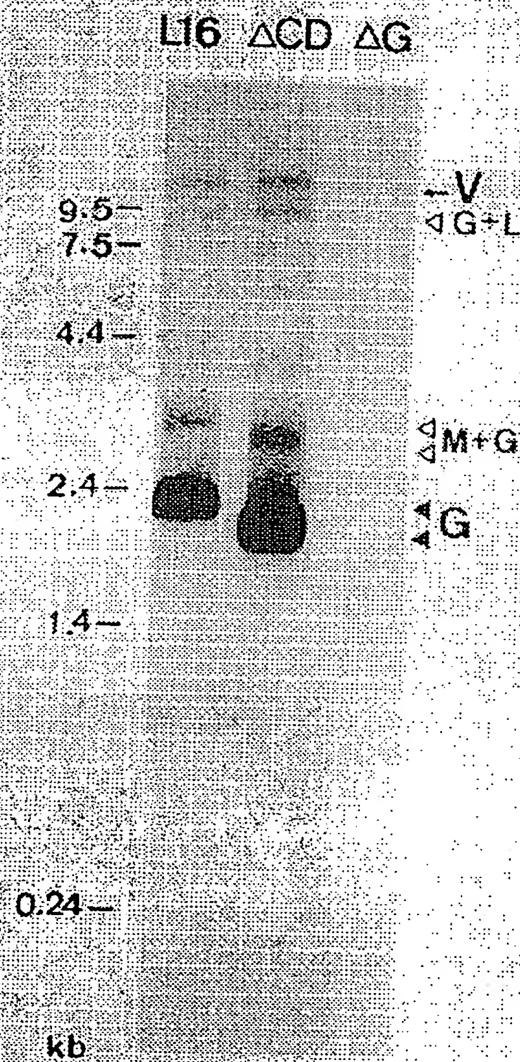

FIG. 12B

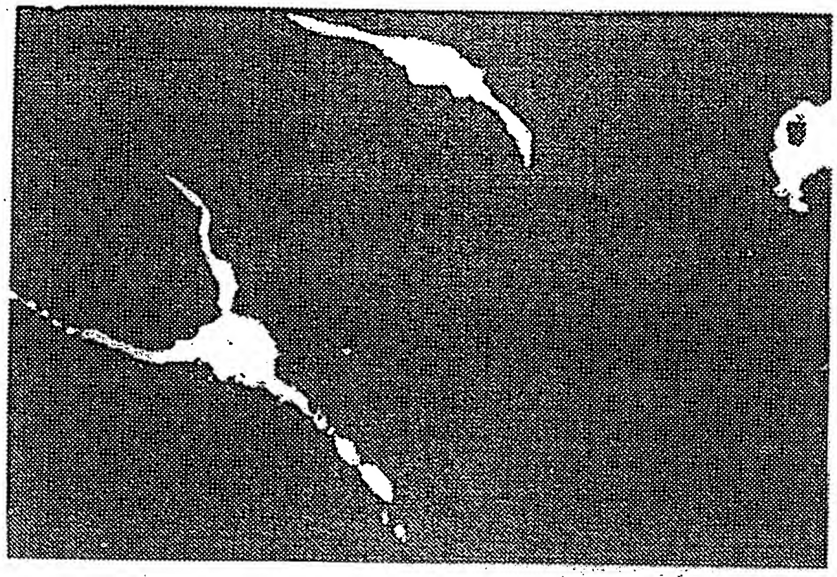

FIG. 13A

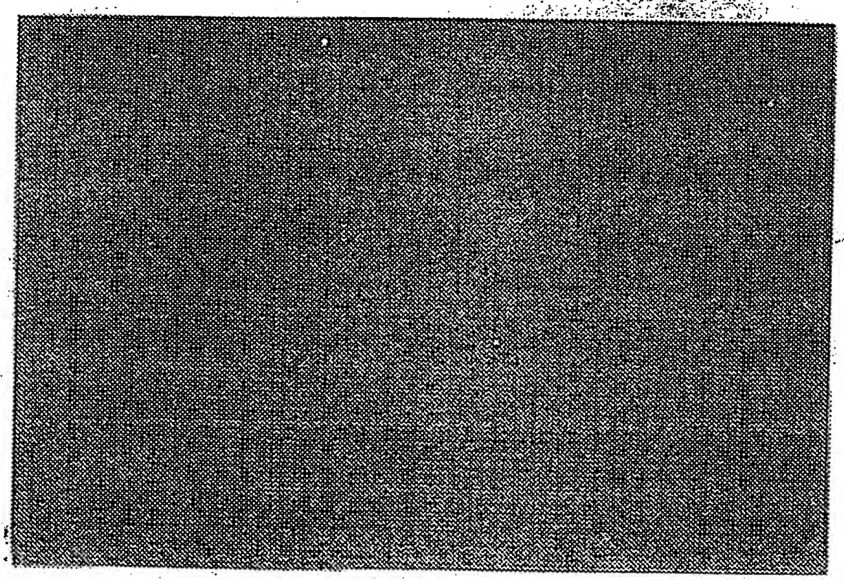

FIG. 13B

```
                transmembrane domain/cytoplasmic domain
...LLSAGALTALMLIIFLMTCC/RRVNRSEPTQHNLRGTGR...  RV-G
...LVGGLRIVFAVLSIVN/RVRpRRVNRSEPTQHNLRGTGR...  HIV/RV-gp
...LVGGLRIVFAVLSIVN/RVRQGYSPLSFQTHLPIPRGPD...  HIV-gp160
```

FIG. 14

RECOMBINANT INFECTIOUS NON-SEGMENTED NEGATIVE STRAND RNA VIRUS

This is a continuation of application Ser. No. 08/503,351 filed Jul. 18, 1995, now abandoned.

BACKGROUND OF THE INVENTION

The present invention is concerned with a genetically manipulated infectious replicating non-segmented negative-stranded RNA virus mutant and a process for the preparation of such a mutant.

Rabies virus (RV) is an example of a non-segmented negative-stranded RNA virus of the Rhabdoviridae family. Other species belonging to this family are vesicular stomatitis virus (VSV), infectious hematopoietic necrosis virus (IHNV) viral haemorrhagic septicaemia virus (VHS, Egtved virus), bovine ephemeral fever virus (BEFV), and sonchus yellow net virus (SYNV).

Beside the family of Rhabdoviridae also viruses belonging to the Paramyxoviridae (e.g. sendai virus (SV), parainfluenza virus (PIV) type 2 and 3, Newcastle disease virus (NDV), mumps virus (MUV), measles virus (MEV) and canine distemper virus (CDV)) and Filoviridae, and several viruses not assigned to a family (e.g. Borna disease virus; BDV) have a nonsegmented negative-stranded RNA genome.

The overall genomic organisation in the non-segmented negative-stranded RNA viruses of the various families is comparable. Especially between the paramyxoviridae and the rhabdoviridae, there are only minor differences in the overall genomic organisation (Tordo et al., Seminars in Virology 3: 341–357, 1992).

RV can infect all warm-blooded animals, and in nearly all instances after establishment of symptoms the infection ends in death. Dog rabies is still important in many parts of the world: infected dogs cause most of the estimated 75,000 human rabies cases that occur each year world-wide. In many countries of Europe, and in the United States and Canada, wildlife rabies has been increasing in importance.

The clinical features of rabies are similar in most species, but there is great variation between individuals. Following the bite of a rabid animal the incubation period is usually between 14 and 90 days, but may be considerably longer, and incubation periods of over a year have been documented. Two clinical forms of the disease are recognized furious and dumb or paralytic. In the furious form, the animal becomes restless, nervous, aggressive, and often dangerous as it loses all fear of humans and bites at anything that gains its attention. The animal often cannot swallow, giving rise to the synonym for the disease, "hydrophobia". There is often excessive salivation, exaggerated responses to light and sound, and hyperesthesia. As the encephalitis progresses, fury gives way to paralysis, and the animal manifests the same clinical features as seen throughout in the dumb form of the disease. Terminally, there are often convulsive seizures, coma, and respiratory arrest, with death occurring 2–7 days after the onset of clinical signs.

Rabies virus enters the body in the bite or occasionally the scratch of a rabid animal, or when virus-loaded saliva from a rabid animal enters an open wound. Viral replication in the bite site, in muscle, is followed by invasion of peripheral nerve endings and central movement of viral genome in the cytoplasm of axons to the central nervous system. Viral entry into the spinal cord and then the brain (particularly the limbic system) is associated with clinical signs of neuronal

dysfunction. Usually, at about the same time that central nervous system infection causes fury, virions are also shed from the apical end of mucus-secreting cells in the salivary glands and are delivered in high concentrations into saliva.

Throughout the course of rabies, host inflammatory and specific immune responses are only minimally stimulated; the most likely reasons for this are because the infection is noncytopathic in muscle and in nerve cells and because the infection is largely concentrated in the immunologically sequestered environment of the nervous system.

RV virions like all Rhabdoviruses are composed of two major structural components: a nucleocapsid or ribonucleoprotein (RNP) core and an envelope in the form of a bilayer membrane surrounding the RNP core. The infectious component of all Rhabdoviruses is the RNP core. The genomic RNA is of negative sense and thus cannot serve as a messenger but requires its own endogenous RNA polymerase for transcription of mRNA. The RNA genome is encapsidated by the nucleocapsid (N) protein in combination with two minor proteins, i.e. RNA-dependent RNA polymerase (L) and phosphoprotein (P) to form the RNP core. The membrane component contains two proteins: an transmembrane glycoprotein (G) and a matrix (M) protein located at the inner side of the membrane. The G-protein is responsible for cell attachment and membrane fusion in RV, and additionally is the main target for the host immune system.

During transcription, the genome directs the sequential synthesis of a short leader RNA and five monocistronic, capped and polyadenylated mRNAs. During replication, the conditional transcription stop and start signals between the cistrons are ignored by the viral polymerase. For both the transcriptase and the replicase reaction the presence of the N-protein complexed with the RNA genome as well as the L- and P-proteins are required. The gene order on the RV genome has been determined and is 3'-leader-N-P-M-G-L-5' as shown in FIG. 1. Each of the mRNAs of RV is translated immediately after transcription. Two events—occur sequentially during replication: first the production of an encapsidated complete positive strand RNA complementary to the genome, followed by the production of complete negative-stranded RNA which is also encapsidated by the N, L and P proteins. Finally, the newly assembled RNP cores associate with M-protein and G-protein during the assembly and budding process leading to the release of fully formed and infectious RV virions.

The 11.9 kb genomic RV RNA contains five open reading frames (ORFs) coding for the N, P, M, G and L proteins, in addition to the presence of a pseudogene region (ψ) between the G and L genes (FIG. 1).

Current vaccines for non-segmented negative strand RNA viruses comprise chemically inactivated virus vaccines or modified live virus vaccines comprising an attenuated virus strain the pathogenicity of which is decreased by multiple passages in cell culture. Chemically inactivated rabies vaccines are e.g.: Rabivac, Behringwerke (human), HDC, Rhone-Poulenc (human), Bayovac-LT, Bayer (vet), Madivac, Hoechst (vet), Epivax-LT, Pitman-Moore, Rabisin, Rhone-Merieux. For RV examples of such attenuated viruses are the vaccine strains SAD B19 and ERA. Inactivated vaccines generally induce only a low level of immunity, requiring repeated immunizations. Furthermore, the neutralization inducing antigenic determinants of the pathogens may become altered by the inactivation treatment, decreasing the protective potency of the vaccine.

In general, attenuated live virus vaccines are preferred because they evoke an immune response often based on both

humoral and cellular reactions. However, during cell culture passaging uncontrolled mutations may be introduced into the viral genome, resulting in a population of virus particles heterogeneous with regard to virulence and immunizing properties. Over attenuation during passage in cell culture can also be a problem with these vaccines. One must achieve a delicate balance between ensuring that the vaccine is not virulent while making certain that it is still protective. In addition it is well known that such traditional attenuated live virus vaccines can revert to virulence resulting in disease outbreaks in inoculated animals and the possible spread of the pathogen to other animals.

Moreover, a problem with combined live viral vaccines is the mutual influence of the antigenic components resulting in a decrease of the potency of one or more of the constituting components.

Furthermore, with currently administered live attenuated or inactivated RV vaccines it is not possible to determine whether a specific animal is a carrier of RV field virus or whether the animal was vaccinated. Hence, it can be important to be able to discriminate between animals vaccinated with a RV vaccine and those infected with a field virus so as to be able to take appropriate measures to reduce spreading of a virulent field virus. The introduction of for example a serologically identifiable marker can be achieved by introducing a mutation in a gene encoding a (glyco-) protein of RV which normally give rise to the production of antibodies in an infected host animal.

It is desired to introduce a mutation into the RV RNA genome in a controlled manner such that for example the resulting mutant RV is attenuated or comprises a heterologous nucleic acid sequence encoding epitopes of foreign proteins, e.g. immunological marker proteins or antigens of pathogens. Recombinant DNA techniques are already widely used for this purpose with DNA viruses and positive strand RNA viruses. Examples for recombinant DNA viruses: Aujeszky virus (PRV); Adenoviruses; Vaccinia viruses. Examples for recombinant positive-strand RNA viruses: Alphaviruses (Sindbis V., Semliki forest virus: H. V. Huang, C. M. Rice, C. Xiong, S. Schlesinger (1989) RNA viruses as gene expression vectors. Virus Genes 3, 85–91). Picornaviruses (Polio virus, Hepatitis A-virus, Foot- and mouth-disease virus: J. W. Almond and K. L. Burke (1990) Poliovirus as a vector for the presentation of foreign antigens. Semin. Virol. 1, 11–20). Directed genetic manipulation of RNA virus genomes depends on the ability to produce recombinant RNAs which are accepted as a template by the particular RNA-dependent RNA polymerases. Transcripts generated by many standard DNA-dependent RNA polymerases (e.g. T7 RNA polymerase or cellular RNA polymerase II) and mimicking viral genomes are recognized by the polymerases of many positive stranded RNA viruses. This allowed recovery of infectious viruses or replicons from cDNA transcripts and the application of recombinant DNA technology to manipulate these genomes in a site specific manner. Since RNAs corresponding to the genomes of positive stranded RNA viruses may function as mRNA for translation of the viral polymerases, an infectious cycle may be initiated by introduction of the genome analogs into a cell. The template of the polymerases of negative-stranded RNA viruses, however, exclusively is the RNP complex. Moreover, and in contrast to positive stranded RNA viruses, their genomic or antigenomic RNA may not function as mRNA and thus all viral proteins involved in replication and transcription of artificial RNAs have to be provided in trans.

An appropriate system for encapsidation of genomic RNA analogs of a negative-stranded RNA viruses with a segmented genome in order to provide the appropriate template is recently disclosed by Palese, P. et al., (WO 91/03552). RNA transcripts from influenza virus genome segments were encapsidated by purified proteins in vitro which can be used to transfect cells together with a helper virus. However, it was found that this approach was not successful with RV, a virus having a non-segmented genome. Short model genomes of VSV and RV lacking the major part of the RNA genome comprising the genes encoding the viral proteins could be encapsidated and expressed by plasmid encoded proteins (Pattnaik, A. K. et al, Cell 69, 1011–1020, 1992; Conzelmann, K.-K. and M. Schnell, J. Virology 68, 713–719, 1994). This approach involved the co-expression of both the genome analogs optionally comprising reporter gene inserts, and particular viral proteins from transfected plasmids in order to produce defective virus particles. Ballart et al. described a method to obtain infectious measles virus, also a non-segmented negative-stranded RNA virus, from cloned cDNA (The EMBO Journal, 9: 379–384 (1990)). A European Patent Application relating to this method was filed with the author as one of the inventors.

Both the paper and the Application were withdrawn however, since further research revealed that all supposed recombinant viruses were no recombinants at all, but mere progeny virus of the originally used vaccine strain.

Thus it must be concluded, that attempts to obtain infectious recombinant negative-stranded RNA viruses with a large, non-segmented genome which necessitates manipulation of the entire genomes, have failed until now.

SUMMARY OF THE INVENTION

The present invention provides a genetically manipulated infectious replicating non-segmented negative-stranded RNA virus mutant, obtainable by recombinant DNA techniques, comprising an insertion and/or deletion in an ORF, pseudogene region or non-coding region of the RV genome.

More specifically the invention provides non-segmented negative-stranded RNA viruses of the paramyxo- and rhabdovirus family.

As explained above, there is a large homology in genomic organisation between the non-segmented negative-stranded RNA virus families. Where the function of encoded proteins in the process of replication, assembly, cell attachment or cell fusion is comparable, these proteins will be referred to further as "analogs". It may be that the function of e.g. two proteins of one family is united in one protein in another family. This is e.g. the case with the F and HN proteins of the paramyxoviridae, that together have the same function as glycoprotein G of the Rhabdoviridae. In this case, the two proteins of the one family will be considered analogons of the one protein of the other family.

The insertion and deletion of one or more nucleic acid residues can be introduced in the RV genome by incorporating the appropriate mutations into the corresponding viral ORF, pseudogene region or non-coding region. This alteration is understood to be a change of the genetic information in the RV ORF or pseudogene of a parent RV thereby obtaining the insertion or deletion RV mutant according to the invention.

A mutation, in which one or more nucleotides are replaced by other nucleotides, a socalled substitution replacement is considered to be the result of a combined deletion and insertion action. This kind of mutation is therefore also considered to be included in the wording: deletion and(/or) insertion.

It is clear that any mutation as defined herein comprises an alteration of appropriate RV sequences such that the resulting RV mutant is still infectious and replicating, i.e. the mutant RV is capable to infect susceptible cells and its mutant RNA genome is capable of autonomously replication and transcription, i.e. no co-expression of RV N, P and L proteins is required.

It goes without saying, that also comprised in the present invention are mutant RVs capable of only one single round of infection, followed by replication (Vide infra).

The genomic organisation of different RV strains is identical. The nucleotide sequence and deduced amino acid sequence analysis of the vaccine strain SAD B19 and the virulent strain PV have been determined (Conzelmann et al., Virology 175, 485–499, 1990 and Tordo et al., Nucleic Acids Res. 14, 2671–2683, 1986; Proc. Natl. Acad. Sci U.S.A. 83, 3914–3918, 1986; Virology 165, 565–567, 1988). In Conzelmann et al., 1990 (supra) it is determined that the viral genome of the SAD B19 strain comprises 11.928 nucleotides and that the deduced amino acid sequence of the five viral proteins N, P, M, G and L are highly similar to those of the pathogenic PV strain. The location of the respective ORFs, pseudogene region and intergenic non-coding regions in RV have been determined therein: the coding region of the RV N, P, M, G and L genes correspond with positions 71–1423, 1514–2407, 2496–3104, 3317–4891, 5414–11797, respectively. The pseudogene region (ψ) maps at position 4961–5359, whereas the intergenic regions separating the five cistrons and which are flanked by non-coding sequences containing transcriptional start and stop/polyadenylation signals map to positions 1483–1484; 2476–2480; 3285–3289; 5360–5383. Although the numbering and the nucleotide sequence of the ORFs, pseudogene region or non-coding regions of the parent RV strain used herein to introduce a mutation is not necessarily the same as that of the SAD B19 or PV strain, the above-mentioned characterisations of these regions exactly define the localisation thereof on the genome of any RV strain.

A method to obtain an attenuated RV from a virulent parental RV strain is to introduce the insertion and/or deletion in an ORF encoding a viral protein, for example such that the activity of the viral protein for host cell attachment and membrane fusion is modified, e.g. reduced. It is known for RV that changes in the amino acid sequence of the trans-membrane glycoprotein G have significant effects on the pathogenicity of the RV. In addition, with regard to attenuation also changes in the matrix (M) protein may influence the conformation of the G protein resulting in an attenuation of the virus. Therefore, mutant RV comprising a deletion or insertion in the ORF encoding the G or M protein are particularly preferred herein.

Also comprised in the present invention are infectious replicating rabies virus mutants capable of only one single round of infection, followed by replication. The advantage thereof is explained below:

Although in general recombinant live vaccines have been proven to be safe and efficacious, there is a risk that the vaccine viruses may spread to other animals which are more susceptible to the virus.

Therefore, there is a strong reluctance on both political, ethical and partially scientific grounds, to allow the use of recombinant viruses in the field.

In particular, for risk assessment studies by regulatory authorities with respect to genetically modified vaccine viruses, especially live viruses expressing foreign genes, the aspect of possible shedding of these viruses in the environment is a very important aspect.

Thus, it can be appreciated that rabies virus vaccines which display all the advantages of live virus vaccines but which are confined to the vaccinated animals and are not shed, are highly desirable.

Such viruses can be made by e.g. mutation of the M-gene, encoding the M(atrix-)protein. The M-protein plays a main role in the assembly of the virus, whereas it additionally influences the incorporation and conformation of the glycoprotein G.

When $M^{(-)}$ mutants, lacking a functional M-protein, are grown in manipulated cells that produce the M-protein in trans, intact virus particles are made, that behave like wild-type virus as far as their infectious character towards their natural host is concerned. Once they have infected a host cell however, there is no possibility to form new infectious viruses, since they lack the genetic information to synthesize the M-protein.

Therefore, they remain contained in the host. The advantages of such viruses will be discussed below.

Therefore, in a preferred embodiment the present invention relates to an insertion and/or deletion in the open reading frame encoding the matrix protein M, such that it results in a non-functional matrix protein M, or even in the absence of matrix protein M. The $M^{(-)}$ mutant viruses with the non-functional or absent matrix protein M have to be grown in cells that provide a matrix protein M analog in trans, in order to phenotypically complement the virus.

Alternatively, such viruses can be made by e.g. mutation of the G-gene. The G-protein plays a main role early in infection, in the process of cell attachment and membrane fusion, as mentioned before.

It is possible to mutate the G-gene by insertion and/or deletion (or even by deletion of the whole G-gene) to such an extent that the resulting G^- mutant virus is no longer capable of successfully infecting other cells, due to heavily impaired (or even absent) glycoprotein G. Such mutants will further be referred to as G-minus (G^-) mutants.

This kind of mutations of the G-gene is therefore more severe than the mutation described before, that only lead to decreased virulence: complete G^- mutants are not infectious, since they lack a functional glycoprotein G.

If such G^- mutant viruses are grown in recombinant host cells complementing for the G-protein, progeny viruses are excreted that are phenotypically G-positive, but genotypically G-negative.

These viruses have an important advantage over G-positive viruses: on the one hand, they are capable of infecting non-complementing host cells, since they possess the G-protein in their membrane. In the infected cells, the G^- mutant viruses replicate as wild-type viruses. This has the advantage that the whole viral genome, including heterologous genes cloned into the recombinant virus, is multiplied, and the encoded genome products will be expressed and processed as with wild-type virus.

On the other hand however, no infectious progeny virus can be made in the host, since normal host cells do not synthesize G-protein, and the mutant virus itself is genotypically G-negative.

Thus, animals infected with G^- mutant virus do not shed infectious virus in the environment. This makes G^- mutants (as well as the $M^{(-)}$ mutants discussed above) very safe as a basis for vaccines.

Alternatively, the G^- mutants according to the invention can be complemented phenotypically by other, non-rabies-, glycoproteins known to play a role in cell attachment.

Since glycoprotein(s) protruding from the viral membrane into the environment are known to determine the cell-specificity, it therefore is possible to target the recombinant infectious rabies virus mutant to specific cells other than the natural host-cells of rabies, by chosing the right complementing glycoprotein.

These glycoproteins will further be called "glycoprotein G analogs", to indicate that they are involved in cell-specific attachment, like glycoprotein G.

It should be noticed, that in some viruses, the "glycoprotein G analogs" determining the cell specificity are not glycoproteins but non-glycosylated proteins. It is clear, that these proteins are also within the scope of the invention.

Therefore, in another preferred embodiment of the present invention, the insertion and/or deletion in the open reading frame encoding the glycoprotein G is such that it results in a non-functional glycoprotein G, or even in the absence of glycoprotein G. The $G^{(-)}$ mutant viruses with the non-functional or absent glycoprotein G have to be grown in cells that provide a glycoprotein G analog in trans, in order to phenotypically complement the virus.

In an even more preferred embodiment of the present invention, the glycoprotein analog used for complementation is the rabies virus glycoprotein G itself.

Recombinant infectious rabies viruses with a glycoprotein G analog have several important advantages:

a) they can be specifically targeted to certain cells, organs or hosts, depending on the target of the glycoprotein G analog that was chosen,

This implies that e.g. specifically the respiratory tract or the digestive tract can be targeted. Thus, e.g. mucosal responses can be obtained at a predetermined site.

Alternatively, specific cells of the immune system can be targeted.

b) they can additionally be carriers of foreign genetic information encoding epitopes from non-rabies pathogens as explained above.

Alternatively, they can be carriers of foreign genetic information encoding toxic substances.

A very important application of viruses according to the invention is obtained with viruses having both a glycoprotein G analog according to a) and foreign genetic information according to b).

Recombinant infectious rabies viruses can be obtained according to the present invention, that are targeted to a specific cell type, normally attacked by a non-rabies virus, while at the same time carrying an immunoprotective determinant of that non-rabies virus.

Such a virus induces immunity in the host against the non-rabies virus, whereas at the same time it is fully safe, due to the lack of genetic information for the glycoprotein G analog.

Another important embodiment of the present invention are viruses according to the present invention that are e.g. targeted to CD4-cells, that represent target cells of HIV, through genotypical complementation with HIV gp 120, and that facultatively encode a cytotoxic protein.

Such viruses will selectively attack CD4-cells, and once inside these the cells, they will kill them.

Alternatively, recombinant infectious rabies viruses according to the present invention can provide very safe vaccines against virulent/pathogenic viruses against which at this moment no safe, live vaccines exist: a recombinant infectious rabies virus targeted against e.g. the natural target cells of Bovine Respiratory Syncytial Virus (BRSV) through complementation with BRSV glycoprotein G analog, and expressing immunoprotective epitopes of BRSV, gives a very safe vaccine against this disease.

Parainfluenza virus vaccines have so far faced the same problems as BRSV-vaccines. Therefore, recombinant infectious rabies virus with parainfluenza glycoprotein G analog and additional immunogenic epitopes of parainfluenza provides a good and safe vaccine against this disease.

Other important veterinary vaccines based on recombinant infectious rabies virus are made by introduction into the recombinant rabies virus of immunogenic determinants of

i) the toroviruses; equine, bovine and porcine torovirus,

ii) the coronaviruses; bovine, canine, porcine and feline coronavirus, especially the spike-proteins thereof.

Therefore, a most preferred embodiment of the present invention relates to recombinant infectious rabies virus glycoprotein $G^{(-)}$ mutants, complemented with a glycoprotein G analog, and carrying a heterologous nucleic acid sequence encoding an epitope or polypeptide of a pathogenic virus or microorganism.

Alternatively, attenuation of the RV may be obtained by altering the enzyme activity of the RV replicase or transcriptase so that the enzyme is less active, thereby resulting in the production of less infectious virions upon infection of a host animal. As the N, P and L proteins are involved in the RV polymerase activity, RV mutants having an insertion or deletion in the ORF encoding the N, P or L proteins are also part of the invention.

RV deletion and/or insertion mutants according to the invention can also be used to vaccinate a host in order to be able to discriminate (serologically) between a host to which a vaccine comprising said RV mutant is administered and a host infected with a parental RV. In this embodiment of the invention the insert in the RV insertion mutant may encode a heterologous epitope which is capable of eliciting a specific non-RV immune response in an inoculated host, or may encode a protein with enzymatic activity, such as CAT or lacZ (Conzelmann and Schnell, 1994, supra). A preferred region for the incorporation of such inserts is the RV pseudogene region. As is demonstrated in the Examples insertions and deletions can be made in this region without disrupting essential functions of RV such as those necessary for infection or replication. The RV deletion mutant may lack an epitope of a RV protein against which an immune response is normally raised by the vaccinates, in particular a RV mutant comprising a deletion in the ORF encoding the G protein is suited for this purpose. In the case of a RV insertion mutant the insertion comprises a nucleic acid sequence encoding a serological marker antigen or an epitope thereof.

In a further embodiment of the invention a RV mutant is provided which is capable of expressing one or more different heterologous epitopes or polypeptides of a specific pathogen.

Such a mutant can be used to vaccinate animals, both domestic and non-domestic animals, against wildlife rabies and said pathogen.

Vaccination with such a live vector vaccine is preferably followed by replication of the RV mutant within the inoculated host, expressing in vivo the heterologous epitope or polypeptide along with the RV polypeptides. The polypeptides expressed in the inoculated host will then elicit an immune response against both RV and the specific pathogen. If the heterologous polypeptide derived from the specific pathogen can stimulate a protective immune response, then the animal inoculated with the RV mutant according to the invention will be immune to subsequent infection by that pathogen as well as to infection by RV. Thus, a heterologous

nucleic acid sequence incorporated into a suitable region of the RV genome may be continuously expressed in vivo, providing a solid, safe and longlasting immunity to the pathogen.

In particular, the present invention provides a RV vector which comprises an insertion of a nucleic acid sequence encoding an epitope or polypeptide of a specific pathogen, wherein the insertion is made in the pseudogene region.

If desired, part or whole of the pseudogene region can be deleted in the RV vector described above.

Preferably nucleic acid sequences encoding an epitope or polypeptide of canine parvovirus, canine coronavirus and classical swine fever virus (CSFV) are contemplated for incorporation into a suitable region of the RV genome.

The possibility to manipulate the non-segmented negative-stranded RNA genome of RV on the DNA level by recombinant DNA techniques was not possible until now because no infectious replicating virus could be generated. However, a process is provided herein which allows the engineering of a mutation into a coding region or non-coding region of the viral genome on the DNA level by means of recombinant DNA techniques followed by the generating of an infectious replicating RV harbouring the mutation in its genome.

This process according to the invention comprises the steps of

a) introducing into cells expressing a RNA polymerase;
   1) one or more DNA molecules encoding the RV N, P and L proteins, and
   2) a DNA molecule comprising the RV cDNA genome and
b) isolating the viruses produced by the cells.

Normally, the cDNA of the rabies virus genome is modified by the incorporation of a mutation in the genome.

The process may however also be used to e.g. purify contaminated RV pools. In that case, the original non-mutated cDNA will be used.

In view of the fact that rescue efficiency of a model mini-genome of RV comprising heterologous inserts with plasmid encoding proteins is extremely low and moreover correlates with insert length (Conzelmann and Schnell, 1994, supra) it could not be expected that initiation of a productive infection from transfected full length genomic RNA could be achieved by co-transfection with plasmids encoding the RV N, P and L proteins. This is the more so as large amounts of positive sense N, P and L specific RNAs are produced from the transfected protein encoding plasmids which were expected to hybridize with simultaneously expressed negative-stranded genomic RNA transcripts. Possible hybridization, however, which could affect more than half of the genome was suspected to interfere with the crucial encapsidation step. In addition, translation of N, P and L mRNA might be affected. Indeed, it was found that with the standard transfection protocol no infectious viruses could be obtained. However, as demonstrated in the examples the application of an alternative transfection protocol in combination with the use of a RV cDNA genome generating positive stranded antigenomic RNA transcripts, gave rise to a replicating genetically engineered RV.

The above-mentioned process allows the in vitro incorporation of a mutation in the genome of a parental RV by means of recombinant DNA techniques followed by the generation of an infectious replicating RV mutant harbouring said mutation. The mutation includes but is not limited to an insertion, deletion or substitution of nucleic acid residues into an ORF encoding a RV protein, a non-coding region e.g. the pseudogene region, or a transcriptional signal sequence of RV parental genome.

The engineering of a mutation in a non-coding intergenic region may influence the transcription of a specific viral gene such that the transcription of the mRNA and the subsequent translation of the protein, either an envelope protein, such as the M and G protein or a protein involved in polymerase activity, such as the N, P or L protein, is reduced resulting in a virus mutant featuring attenuated characteristics because the mutant's capability of producing (infectious) progeny virus is reduced. In particular the substitution of one or more nucleic acid residues in this intergenic region and/or transcriptional signal sequences can influence efficiency of transcription.

Furthermore, the substitution of one or more nucleic acid residues in a region of the genome of a virulent RV which is involved with virulence, such as the ORF encoding the G protein, by the application of the process described herein is part of the invention.

Such a mutation may result in the exchange of a single amino acid in the G protein of a virulent RV strain resulting in a (partial) loss of pathogenicity, e.g. replacement of Arg (333) with Ile, Glu or Gln, or Leu (132) by Phe, or Trp.

In the process according to the invention the DNA molecule containing the RV genetic information preferably comprises a plasmid provided with appropriate transcription initiator and terminator sequences recognizable by a polymerase co-expressed by the transfected host cells.

A preferred process according to the invention comprises the use of host cells transfected with RV DNA, said cells being able to express bacteriophage T7 DNA-dependent RNA polymerase, expressed for example cytoplasmically from vaccinia virus recombinant. In this case the plasmids containing RV DNA are provided with the T7 promoter and terminator sequences (Conzelmann and Schnell, 1994, supra).

For the preparation of a live vaccine the recombinant RV mutant according to the present invention can be grown on a cell culture derived for example from BHK, or human diploid cells. The viruses thus grown can be harvested by collecting the tissue cell culture fluids and/or cells. The live vaccine may be prepared in the form of a suspension or may be lyophilized.

In addition to an immunogenically effective amount of the recombinant RV the vaccine may contain a pharmaceutically acceptable carrier or diluent.

Examples of pharmaceutically acceptable carriers or diluents useful in the present invention include stabilizers such as SPGA, carbohydrates (e.g. sorbitol, mannitol, starch, sucrose, glucose, dextran), proteins such as bovine serum or skimmed milk and buffers (e.g. phosphate buffer).

Optionally, one or more compounds having adjuvant activity may be added to the vaccine. Suitable adjuvants are for example aluminium hydroxide, phosphate or oxide, oil-emulsions (e.g. of Bayol F(R) or Marcol 52(R)), saponins or vitamin-E solubilisate.

The useful dosage to be administered will vary depending on the type of mammal to be vaccinated, the age, weight and mode of administration.

The dosage may vary between wide ranges: 10^2 to 10^7 pfu/animal would e.g. be suitable doses.

A specific dosage can be for example about 10^6 pfu/animal.

A RV mutant according to the invention can also be used to prepare an inactivated vaccine.

For administration to animals, the RV mutant according to the present invention can be given inter alia orally, intranasally, intradermally, subcutaneously or intramuscularly.

The RV vaccine according to the invention can be administered to dogs but also to the main vectors, i.e. raccoons, skunks and foxes. Furthermore, also vaccination of wild boars with a live RV vector capable of expressing a heterologous gene of a porcine pathogen such as classical swine fever virus, is contemplated.

EXAMPLE 1

Preparation of Infectious Replicating RV Virions

Construction of Full Length RV cDNA (FIG. 2).

The cloning of cDNA spanning the entire genome of RV strain SAD B19 was described previously (Conzelmann et al., 1990, supra; GenBank accession number M31046). The numbering of RV nucleotides and amino acids used herein corresponds to that of Conzelmann et al., 1990 (supra). As basis for the assembly of a SAD B19 full length DNA clone the RV mini-genome sequence contained in the transcription plasmid pSDI-1 (Conzelmann and Schnell, 1994, supra) was used (FIG. 2). pSDI-1 contains the SAD B19 genomic 3' and 5' ends (SAD B19 nucleotides 1–68 and 11760–11928, respectively) inserted between a T7 RNA polymerase promoter and the hepatitis delta virus (HDV) antigenome ribozyme sequence. In order to generate a plasmid to produce positive stranded SDI-1 transcripts (pSDI-1plus) the RV sequences contained in pSDI-1 were first amplified by PCR using an 11 base primer (5'-ACGCTTAACAA-3') (SEQ ID NO:1) which due to the complementarity of RV genome ends corresponds to the 5' termini of both positive and negative sense viral RNAs. After subsequent partial ligation of a synthetic EcoRI/blunt adaptor (T7/3) containing a T7 promoter sequence followed by three G residues (underlined) (5'-AATTCCTGCAGTAATACGACTCACTATAGGG-3') (SEQ ID NO:2) to the amplified RV sequence, the ligation products were cloned in the EcoRI/SmaI sites of pX8dT. This plasmid is a derivative of pBluescriptII (Stratagene) from which a BssHII/ClaI fragment of the multiple cloning site containing the original T7 promoter was deleted. It contains the 84 base HDV antigenomic ribozyme sequence in the SmaI site followed immediately by a T7 transcription terminator sequence cloned in the BamHI site. Constructs that contained a T7 promotor upstream of the plus sense RV sequence were identified by restriction analysis and sequencing. The MunI-BglII fragment of pSDI-1 (SAD B19 nucleotides 40–68) was then replaced with a 1 kb MunI/BglII cDNA construct assembled in pBluescriptII from three fragments of different SAD B19 cDNA clones (MunI-SphI (SAD B19 nucleotides 40–482 from pZAD1-9); SphI-AatII (4041–4273 from pSAD13), and AatII-BglII (11472–11759 from pSAD85)) resulting in pSDI-1170. By insertion of a SphI fragment assembled from the clones pSAD25 and pSAD13 via NcoI (SAD B19 nucleotides 482–4041) and an AatII fragment assembled from clones pSAD 49 and pSAD85 via XhoI (SAD B19 nucleotides 4273–11472) into the unique SphI and AatII sites of pSDI-1170, the final basic full length clone pSAD L16 was completed. Using the circular plasmid, in vitro transcriptions were performed and the products analyzed on denaturing agarose gels. The presence of RNA transcripts co-migrating with 12 kb RV genomic RNA indicated that full length antigenome RNA is transcribed by T7 polymerase.

Recovery of Infectious Recombinant RV

The co-transfection of plasmid pSAD L16 and plasmids encoding RV proteins N, P and L was carried out as described in Conzelmann and Schnell, 1994 (supra).

Transfection experiments were carried out as described previously. BHK-21, clone BSR cells were grown overnight in 3.2 cm-diameter dishes in Eagle's medium supplemented with 10% calf serum to 80% confluence, and infected at a m.o.i. of 5 with the recombinant vaccinia virus vTF7-3 (Fours et al., Proc. Natl. Acad. Sci U.S.A. 83, 8122–8126, 1986). One hour post-transfection cells were washed twice with culture medium lacking calf serum and transfected with a plasmid mixture containing 5 μg pT7T-N, 2.5 μg pT7T-P, and 2.5 μg pT7T-L and with 2 μg of pSAD-L16 plasmid by using the mammalian transfection kit (Stratagene; $CaPO_4$ protocol) according to the suppliers instructions. The precipitate was removed 4 h posttransfection and cells were washed and incubated in Eagle's medium containing 10% calf serum. Possible encapsidation of pSAD-L16 derived T7 RNA polymerase transcripts and the resulting expression of RV proteins from the nucleocapsids was checked by indirect fluorescence. A monoclonal antibody directed against RV G protein, which could only be expressed from the recombinant RV genome, was used to screen the cultures. One day after transfection stained cells were present, demonstrating expression of genes from the RV genome. However, only single positive cells were observed in a series of 20 transfection experiments. No fluorescent cell foci indicating the presence of infectious virus were obtained in these experiments. In addition, from cell cultures which were inoculated with the entire supernatant from the transfected cells no infectious virus could be recovered two days later. Therefore, in order to isolate a presumed very low number of infectious virus generated in transfected cells, the experimental procedure was modified. For isolation of transfectant viruses cells and supernatants were harvested 2 days post transfection. Cells were suspended in the supernatant by scratching with a rubber policeman. The suspension was submitted to three cycles of freezing and thawing (−70° C./37° C., 5 min each). Cellular debris and the excess of vaccinia virus which forms aggregates under these conditions was pelleted by 10 min of centrifugation at 10.000 g in a microfuge. The entire supernatant was used to inoculate a culture dish with a confluent monolayer of cells. After incubation for 2 h, the supernatant was replaced by 2 ml of fresh culture medium. A cythopathogenic effect (cpe) caused by vaccinia virus was observed one to two days post infection. In average only ten plaques were observed after centrifugation at 10.000 g. RV infection of cells, which does not result in detectable cpe was demonstrated two days post infection by direct immunofluorescence staining of the entire monolayer with an anti-N conjugate (Centocore). In two out of 20 experiments fluorescent foci were observed and the respective supernatants contained infectious RV (SAD L16) which was assumed to represent transfectant virus generated from cDNA transcripts.

Half of the supernatants from the cultures in which foci were observed, was used for the second passage after centrifugation at 10.000 g. For further passaging (2 days each) decreasing aliquots of supernatants were used according to the degree of RV infection. To get completely rid of Vaccinia virus, supernatants from cultures approaching infection of all cells (third passage) were centrifuged two times for 10 min at 14.000 g in a microfuge. The final supernatant was then filtered using a sterile MILLEX-VV 0.1 μm filter unit (Millipore Products, Bedford, Mass. 01730) and then used to produce high titre stocks of recombinant RVs.

The latter transfection and isolation protocol was used in the subsequent Examples.

EXAMPLE 2

Insertion of an Oligonucleotide in the RV Pseudogene Region

Manipulations of the ψ were carried out in the sub-clone pPsiX8, containing a 2.8 kb XhoI-ScaI fragment of pSAD

L16 representing SAD B19 nucleotides 3823 to 6668. The StuI fragments of the modified pPsiX8 plasmids were then isolated and used to replace the corresponding fragment (SAD B19 position 4014 to 6364) of the full length clone pSAD L16 (FIG. 1). Insertion of 4 nucleotides into the ψ and generation of a novel NheI site was achieved by digestion of pPsiX8 with Hind III, fill in of the extensions with Klenow enzyme and religation. The final full length clone pSAD U2 is distinguished from SAD L16 by the duplication of nucleotides 5338 to 5341.

The generation of infectious viruses was demonstrated after transfer of extracts from transfected cells together with supernatant to fresh cells. In each of the series focus formation was observed in one experiment. The transfectant viruses (clones SAD U2-13 and SAD U2-32) were passaged by transfer of supernatants to fresh cells two further times resulting in almost 100% infection of the cells. To demonstrate the insertion in the SAD U2 virus genome, total RNA was isolated from cells infected with SAD U2-13 and reverse transcriptase-PCR (RT-PCR) of the ψ was performed. With the primers G3P and L4M (FIG. 1), which are specific for the G and L genes, respectively, DNA fragments of approximately 730 bp were obtained from the genomes of transfectant viruses SAD U2 and SAD L16 and of standard RV SAD B19. However, subsequent digestion with HindIII was only observed for the PCR DNA obtained from SAD B19 and SAD L16, but not for that from SAD U2. Conversely, only SAD U2 derived DNA was digested with NheI, giving rise to two fragments of approximately 530 and 200 bp, respectively (FIG. 3). Direct RT sequencing of genomic RNA of transfectant virus SAD U2 further confirmed the presence of the expected insertion of 4 residues at the predicted site, while the rest of the determined sequence corresponded to that of the original SAD B19 genome. Thus, it was clear that SAD U2 virus represented a transfectant virus whose genome originated from engineered cDNA.

The introduction of four additional nucleotides close to the end of the RV ψ did not affect viability of the transfectant virus SAD U2, nor did it interfere with correct transcription termination of the G mRNA.

EXAMPLE 3

Alteration of RV Transcription by an Insertion or Deletion Between G and L Coding Region

By double digest with StyI and HindIII, Klenow fill in and religation, 396 bases (SAD B19 nucleotides 4942 to 5337) were deleted, the final construct was pSAD W9. For the construction of pSAD V*, a 180 bp BglII-AsuII fragment including the SAD B19 N/P cistron border region was isolated from pSAD13 (Conzelmann et al., 1990, supra). The fragment contained 97 nucleotides of the N coding region, the entire 3' non-coding region and the N/P cistron border consisting of the N transcriptional stop/polyadenylation signal, the intergenic region, and the first 16 nucleotides of the P cistron including the transcriptional start signal. The cDNA fragment was first sub-cloned into the EcoRI site of pBluescript after fill-in of 3' recessive ends with Klenow enzyme (pNigP-180). After excision with HindIII/XbaI from pNigP and blunt end generation the obtained 230 bp fragment which contained the RV insert flanked by 16 and 34 bp of vector derived sequences, respectively, was cloned into the filled-in StyI of pPsiX8. The final full length construct (pSAD V*) thus possessed a 234 bp insertion compared to pSAD L16.

As before, pSAD V* and pSAD W9 were used to transfect twenty culture dishes each. In three cultures transfected with SAD V* and in one with SAD W9, rescue was indicated by subsequent isolation of viable virus. After five successive passages RNA from infected cells and supernatant was isolated and analyzed by RT-PCR using the same primers as in the previous experiments. In comparison to standard SAD B19 virus, an enlarged DNA fragment of approximately 0.9 kb resulted from RNA of cells infected with SAD V* thus showing that additional sequences were present in the ψ region of this transfectant virus (FIG. 4). In contrast, from RNA of cells infected with SAD W9, a DNA fragment of only 0.3 kb was obtained; this size was expected according to the deletion made in the cDNA genome copy. Sequencing of PCR products confirmed further that the original engineered cDNA sequences were rescued into the genomes of SAD V* and SAD W9 transfectant viruses. Accordingly, neither the presence of additional sequences, including 50 vector derived nucleotides, between the G open reading frame and the ψ nor the deletion of the entire ψ did interfere with the infectivity and propagation of transfectant rabies viruses. The alterations engineered into the genomes of SAD V* and SAD W9 were designed in a way to result in phenotypical changes in the transcription pattern and it was investigated whether this affected the growth characteristics of the respective transfectant viruses. However, propagation in cell culture as well as final titers of infectious SAD V* and SAD W9 viruses were similar to those of standard SAD B19 RV. Three days after infection of cells with an m.o.i. of 0.01, titers of 10^8 focus forming units (ffu) were reached in the supernatants for SAD B19, SAD V* and SAD W9 demonstrating that the RV ψ is not essential for propagation in cell culture.

Using a ψ specific probe, no hybridization was detected with RNA from cells infected with the ψ-deleted SAD W9 virus. While the genomic RNAs of the other viruses and the G mRNAs of SAD B19 and SAD L16 were recognized by this probe, the SAD V* G mRNA did not react. In contrast, a faint band of RNA appeared that corresponded in size to the novel extra-ψ-mRNA that was predicted by the presence of the extra P gene transcriptional start signal preceding the SAD V* ψ sequences. In contrast to naturally occurring RV, the transfectant virus SAD V* represents a RV whose genome is composed of six functional cistrons.

EXAMPLE 4

Expression of a Foreign Protein-Encoding Gene from Recombinant RV

The 230 bp cDNA fragment containing the N/P cistron border flanked by multiple restriction sites described in example 3 was introduced into the BstXI site of the pseudogene region of the full length cDNA pSAD L16 (SAD B19 position 4995) after generation of blunt ends with Klenow enzyme. The resulting cDNA pSAD V was used as a basis for introduction of the bacterial chloramphenicol-acetyltransferase (CAT) gene. To obtain pSAD XCAT, a 0.8 kb DNA fragment of pCM7 (Pharmacia) containing the entire CAT coding region was cloned into the AsuII site of pSAD V contained in the N/P cistron border upstream of the pseudogene sequence. For construction of pSAD VCAT, the cDNA between the AsuII site and the HindIII site located close to the end of the pseudogene sequence (SAD B19 position 5337) was deleted and replaced with the CAT-encoding HindIII-DNA from pCM7 after blunt end generation with Klenow enzyme. Accordingly, transcription of the recombinant RV SAD XCAT should give rise to a CAT mRNA possessing the pseudogene sequence as a nontranslated 3' region, whereas SAD VCAT should transcribe a CAT mRNA lacking the pseudogene sequence.

Recombinant rabies viruses were rescued after transfection of plasmids encoding RV N, P, and L proteins and

pSAD-XCAT, and pSAD-VCAT, respectively, as described in Example 1. After removal of vaccinia virus, the transcription pattern of the recombinant RVs were analysed by northern hybridization. Both viruses transcribed CAT mRNAs of the expected size and composition (FIG. 5). The expression of CAT enzyme activity was determined in cells infected with the two viruses, respectively, by standard CAT assays (Conzelmann and Schnell, 1994, supra). Both were found to express CAT efficiently. Successive passages in cell culture cells showed that the introduced foreign sequences are genetically stable. Even after 40 passages both viruses expressed CAT efficiently (FIG. 6). Additional experiments were performed in order to examine expression and behaviour of the recombinant viruses in infected animals. Six week old mice (five each) were injected intracerebrally with 10^4 ffu of SAD VCAT, SAD XCAT, and standard sequence RV SAD L16, respectively. Seven days after infection all animals showed typical rabies symptoms and died from rabies within the following week. CAT activity was demonstrated in brains of mice infected with SAD VCAT and SAD XCAT, respectively. Both viruses could be reisolated from mouse brains and expressed CAT cell culture. Thus, a foreign gene can be introduced into the genome of infectious RV and be expressed stably and as well may serve as a marker to differentiate recombinant viruses.

EXAMPLE 5

Expression of a Heterologous Viral Antigen from Recombinant RV and Induction of an Immune Response Against RV and the Heterologous Virus

The genome of classical swine fever virus (CSFV) encodes three structural glycoproteins (E0, E1 and E2). In CSFV infected animals neutralizing antibodies are directed against E2, whereas E0 induces a cellular immune response. cDNA encompassing the coding region of the E2 protein and the E0 protein of CSFV strain Alfort respectively were used to replace the pseudogene region between the AsuII and HindIII sites of pSAD V as described in Example 4. Recombinant viruses (SAD-VE0 and SAD-VE2, respectively) were recovered from transfection experiments as detailed in Example 1. In infected cells the viruses expressed CSFV E0 protein, and CSFV E2 protein, respectively (FIG. 7).

The recombinant viruses SAD VE0 and SAD VE2 were used to immunize pigs by the oral route. Standard fox baits usually being used for oral immunization of foxes with the attenuated RV SAD B19 strain were loaded with 10^7 pfu of SAD-VE0, SAD-VE2 and SAD B19, respectively. Two baits of each preparation were fed to two pigs each (pig #1 and #2: SAD VE0, #3 and #4, SAD B19, #5 and #6, SAD VE2). Four weeks after immunization, the presence of neutralizing antibodies against RV and CSFV as analysed. With the exception of #5, all pigs possessed RV neutralizing antibodies (titre >250) confirming uptake of the vaccine baits. Pig 5 was therefore not further considered. Pig #6 developed CSFV neutralizing antibodies at a titre of >16. As expected, pigs #1 to 4 did not develop CSFV neutralizing antibodies. An intranasal challenge with 10^7 pfu of CSFV strain Alfort was performed 5 weeks after immunization. Leucocyte numbers of pigs and body temperature were monitored after the challenge and shown in FIGS. 8 and 9, respectively. All pigs developed fever, but pigs #1 and #2 as well as #6 recovered more quickly. The control animal #4 died 15 days post challenge with typical CSFV symptoms, the control #3 was killed on day 21. The presence of CSFV neutralizing antibodies in the pig fed with SAD VE2 and the partial protection of the pigs that received either SAD VE0 or SAD VE2 demonstrate that both humoral and cellular immune responses against two heterologous viruses may be induced by recombinant RV live vaccines after application by the oral route.

EXAMPLE 6

Generation of an Attenuated RV by Introduction of a Mutation into G Gene Sequences

In order to generate a virus propagating less efficiently than the standard virus SAD B19, a recombinant was prepared that possesses a mutated G protein.

For this purpose, the sequence encoding the last 46 amino acids of the G protein were deleted. First, the G protein coding plasmid, pT7T-G (Conzelmann and Schnell, 1994, supra) was digested with AflIII (position 4752 of the SAD B19 sequence) and EcoRV (the latter site is present in the multiple cloning site of the plasmid) and blunt ends were generated by Klenow enzyme. Ligation of the resulting AflIII and EcoRV ends resulted in the generation of a translation termination codon at the former AflIII sequence. A 0.3 kb DNA PpuMI-SMaI fragment containing the modified region was used to replace the authentic PpuMI-BstXI fragment 4469–4995 of pSAD L16. This manipulation resulted in the deletion of SAD B19 nucleotides 4753–4995 encoding the carboxyterminal 46 aa of the G protein cytoplasmic tail and part of the pseudogene sequence. A further result is the introduction of 18 vector-derived nucleotides immediately downstream of the new G translation termination codon.

Recombinant RV (SAD DCD) was recovered as described in Example 1. As expected, a truncated G protein was expressed in cells infected with SAD DCD (FIG. 10). Compared to standard sequence virus SAD L16, 100 fold lower titres were obtained with SAD DCD virus after infection of cells at an m.o.i. of 1. In addition, a reduced rate of spread in cell cultures was observed (FIG. 11), indicating that the truncation of the G protein resulted in reduced assembly of virions or reduced cell infectivity of virions. To analyse the behaviour of SAD DCD in infected animals, five mice were injected intracerebrally with 10^5 ffu of SAD DCD and 5 mice with the same dosis of SAD L16.

EXAMPLE 7

Generation of a Rabies Virus G-minus (G^-) Mutant by Complementation in Trans

In order to delete the entire G protein coding region from the RV genome, the full length clone pSAD UE (Example 2) was used. This clone differs from pSAD L16 by the presence of a unique NheI site within the nontranslated 3' region of the G gene (SAD B19 position 5339). By partial digestion of pSAD U2 with PflMI (SAD position 3176) and complete digestion with NheI, subsequent fill-in by Klenow enzyme and religation, a cDNA fragment comprising SAD B19 nucleotides 3177–5339 was removed. The resulting clone pSAD dG was used in transfection experiments to recover recombinant virus. In addition to plasmids encoding N, P, and L proteins, however, a plasmid encoding the G protein was cotransfected with pSAD dG to complement the G deficiency of the viral genome. The resulting virus SAD dG was passaged to cells again transfected with the G encoding plasmid and infected with the vaccinia virus vTF-7-3 to provide G protein.

RNA transcripts of SAD dG were analyzed by Northern blotting experiments. After hybridization with an N specific probe, the SAD dG genome was found to be considerably smaller than the rabies virus wt genome reflecting the cDNA deletion of 2.1 kb. A probe spanning the entire G coding region, however, failed to hybridize with SAD dG RNAs demonstrating the lack of G encoding sequences (FIG. 12). The identity of the deletion was further confirmed by RT-PCR and sequencing.

Phenotypically complemented SAD dG was able to infect noncomplementing BSR cells, to replicate its genome and to

express the genes encoded by the genome. However, it was not able to produce infectious virions and thus, infection could not spread to other cells (FIG. 13) or be transferred by passage of culture supernatants to other cell cultures.

EXAMPLE 8

Complementation of G Mutants by Heterologous Glycoproteins: Directing Virus to Specific Cells

To demonstrate that heterologous surface proteins may be incorporated functionally in the envelope of a recombinant virus, the G-mutant SAD dG was complemented by recombinant viral glycoproteins as described in Example 7 for the rabies virus G. Infectious pseudotype particles were generated that contained the spike proteins from Mokola virus, another member of the Lyssavirus genus, the rhabdovirus vesicular stomatitis virus (VSV; serotype New Jersey, genus vesiculovirus) and from the retrovirus human immunodeficiency virus (HIV-1, strain NL-43).

Expression from transfected plasmids of the authentic Mokola and VSV-G protein and infection of cells with SADdG resulted in the formation of infectious pseudotype viruses. Compared to rabies virus G and the closely related Mokola virus G, however, a reduced titre was observed with VSV-G (10^4/ml in contrast to 10^6/ml). After replacement of the cytoplasmic and transmembrane domain sequence of VSV-G by the corresponding domains of the rabies virus G protein, however, 10^6 infectious particles were generated suggesting that the cytoplasmic domain of the RV G is directing the protein into the viral envelope.

The generation of pseudotype particles containing authentic HIV gp160 (gp 120/40) spikes was not observed. In contrast, expression of a chimeric protein composed of the ecto- and transmembrane domain of the HIV gp fused to the cytoplasmic domain of RV G resulted in the formation of RV(HIV) pseudotypes. This confirmed that the cytoplasmic domain of the G protein is responsible for efficient incorporation of spike proteins into the envelope of rhabdoviruses. The RV(HIV) pseudotype particles successfully infected Vero cells expressing the human CD4 surface protein (T4⁺ cells) but not the control cells expressing CD8 (T8⁺ cells) (cells were obtained from the AIDS Research and Reference Reagent Programme). The pseudotype viruses thus possess the host range and cell specificity of HIV.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1:

Organization of the RV pseudogene region (ψ) and construction of recombinant RV genomes (drawn to scale). Numbers indicate the nucleotide positions in the antigenome sequence of SAD B19. On top, the entire RV genome with its five open reading frames is shown. Mutations were carried out in pPsiX8 containing part of the genome (3823–6668) and reintroduced into the full length clone pSAD L16 by exchange of the StuI fragment (4014–6364). In the detail drawing, coding regions are represented by gray boxes, non-coding sequences as lines. Functional transcriptional signal sequences are indicated by filled bar (stop/polyadenylation) and arrowhead (mRNA transcription start). The non-functional signal-like sequence defining the start of the ψ region is shown by the open bar. Arrows indicate the position of oligonucleotide primers G3P and L4M used for RT-PCR analysis of the ψ region. In SAD U2, fill-in of HindIII extensions resulted in insertion of 4 nucleotides and generation of a unique NheI-site. In SAD V*, a cDNA fragment containing the RV N/P cistron border (SAD B19 nucleotides 1323–1502) was inserted into the StyI site; SAD W9 possesses a deletion of the StyI/HindIII fragment.

FIG. 2:

Simplified scheme for the construction of transcription plasmids containing full length RV cDNA. Numbers refer to nucleotide positions of the SAD B19 RV antigenome sequence (Conzelmann et al., 1990). The plasmid pSDI-1plus which served as a basis for reconstruction of full length RV genomic DNA is a counterpart of pSDI-1 (Conzelmann and Schnell, 1994) containing the SDI-1 RV mini-genome that comprises the terminal nucleotides 1–68 and 11760–11928 in opposite direction with respect to T7 RNA polymerase promoter (T7) and hepatitis delta virus antigenomic ribozyme sequence (HDV). The MunI-BglII fragment of pSDI-1 plus was replaced with a 1 kb cDNA construct that was assembled from three SAD B19 cDNA clones as indicated. Insertion of a 3.6 kb SphI and a 7.2 kb AatII fragment which were assembled from two cDNA clones each resulted in the final plasmid pSAD L16 containing full length SAD B19 cDNA. Transcription of this plasmid by T7 RNA polymerase should yield positive stranded (antigenomic) RNA possessing three extra non-viral G residues at the 5' and a precise 3' end after autolysis of the ribozyme. (T7) T7 promoter; (T7T) T7 transcription terminator; (HDV) HDV antigenomic ribozyme sequence.

FIG. 3:

Demonstration of the genetic tag in the genome of the transfectant virus SAD U2.

Total RNA from cells infected with standard RV SAD B19 (B19) and transfectant viruses SAD L16 (L16) and SAD U2 (U2) was isolated 2 days post infection and used for RT-PCR amplification of the respective ψ regions with primers G3P and L4M. The amplified DNA was separated in a 1% agarose gel directly and after digestion with HindIII and NheI, respectively. A NheI restriction site is present only in DNA derived from SAD U2.M, DNA size marker.

FIG. 4:

PCR analysis of SAD B19 (B19), SAD V* (V*), and SAD W9 (W9) genomes. RT-PCR was performed as described in FIG. 3 with primers G3P and L4M. Amplification products were separated in a 1% agarose gel.

FIG. 5:

Demonstration of CAT-mRNAs transcribed by recombinant RVS.

A Northern blot of total RNA from cells infected with SAD L16 (L16), SAD XCAT (X6) and SAD VCAT (VC 18), was hybridized with probes specific for the G gene (G), pseudogene (Y), and CAT gene, respectively. On the left side the viral genomes (v) and particular mRNAs are indicated. While SAD XCAT transcribes an mRNA containing both CAT and pseudogene sequences ("CATY"), SAD VCAT lacks pseudogene sequences and transcribes an mRNA ("CAT") possessing only CAT sequences. The size of RNA markers are given in kb.

FIG. 6:

CAT activity of SAD XCAT and SAD VCAT after multiple passages in cell culture. Cells were infected with viruses from the particular passages (number of passage as indicated) and equal amounts of cell extracts were analysed for CAT activity two days post infection. In lane "–" extracts from cells infected with SAD L16 were analysed.

FIG. 7:

Expression of E0 and E2 protein by recombinant RVs.

Cells were infected with SAD VE0 (isolates 1, 2, 3) and SAD VE2 (isolates a, b, c), respectively. Two days post infection, cell extracts were separated in PAA Gels under reducing conditions and transferred to nitrocellulose mem-

branes. After incubation with monoclonal antibodies directed against CSFV E0 and E2 protein, respectively, and subsequently with a secondary antibody coupled to alkaline phosphatase, the proteins were visualized by addition of substrate and exposure to an X-ray film. As a control, baculovirus expressed and purified E0 and E2 protein was used (B). In addition, extracts from cells infected with CSFV (V) served for comparison.

FIG. 8:

Leucocytes of pigs immunized with SAD VE0 (#1 and 2), SAD VE2 (#6) and standard rabies virus SAD B19 (#3 and #4), and challenged with CSVF. Leucocyte amounts are given in percent of absolute numbers present prior to challenge (day 0). * (#1, day 10 p.ch.): not done, estimated value.

FIG. 9A:

Body temperature of pigs after CSFV challenge (day 0). Animals immunized with SAD VE0 (#1 and #2) developed mild fever until day 11 (#1) or no fever (#2). Both control animals immunized with SAD B19 (#3 and #4) showed high fever over a long period. #4 died at day 15 post challenge from classical swine fever, due to heavy symptoms, #4 was killed 21 days post challenge.

FIG. 9B:

The animal immunized with SAD VE2 developed mild fever only at days 6 to 8. Controls are the same as in FIG. 9A.

FIG. 10:

Expression of a truncated G protein in cells infected with SAD DCD. BSR cells were infected at an moi of 1 with SAD DCD or SAD L16 and at 16 h post-infection labelled with 50 μCi of [^{35}S]methionine for 3 h. Cell extracts were incubated with an anti-rabies G MAb and aliquots of immunoprecipitated samples were either digested with PNGase F (+PF) in order to demonstrate the protein backbones or mock treated (–) to demonstrate the glycosylated proteins. +TM: infected cells were incubated in the presence of 2 μg/ml tunicamycin for 90 min prior to labelling and during the 3 h labelling period. Proteins were separated on 10% SDS-PAGE and visualized by autoradiography. Cell extracts were analysed as above. L16, SAD L16 virus; ΔCD, SAD DCD mutant virus. M: Protein size markers.

FIG. 11:

Spread of SAD L16 and SAD DCD in cell culture. Culture cells were infected at an m.o.i. of 0.05 with SAD L16 (L16) and SAD DCD (DCD), respectively, and analysed at the indicated times post infection by direct immunofluorescence with a conjugate (Centocor®) directed against rabies virus N protein. A slower spread of infection of neighbouring cells is observed in cells infected with SAD DCD.

FIG. 12:

Analysis of SAD dG (Example 7) and SAD dCD (Example 6) specific RNA's. Total RNA of BSR cells infected with SAD L16 (Example 1), SAD dCD (ΔCD) and phenotypically complemented SAD dG virus (ΔG) at m.o.i.s. of 1 was isolated 2 days post infection and analyzed by Northern hybridization. As demonstrated by hybridization with an N gene specific probe (A), the genome of SAD dG is considerably smaller than the standard rabies virus genome (v), reflecting the 2.1 kb deletion of the G gene. A probe spanning the entire G protein encoding sequence fails to hybridize with SAD dG RNAs. The small deletion of the cytoplasmic domain encoding region in the SAD dCD genome is demonstrated by the appearance of a G mRNA (G) that is shorter than the standard rabies virus G mRNA. v: genomic RNA; N, G: monocistronic mRNAs; N+P, M+G, G+L: bicistronic mRNAs

FIG. 13:

Lack of spread of the G$^-$ mutant SAD dG. BSR cells were infected with phenotypically complemented SAD dG and analyzed 36 hours post transfection by immunofluorescence microscopy. In (A) N protein expression is shown by incubation of cells with a FITC-coupled antibody directed against N protein (Centocore). Only single cells are infected, no spread of virus to neighbouring cells is observed. (B): control with a G specific antibody.

FIG. 14:

Composition of the functional chimeric HIV/RV glycoprotein used for generation of RV(HIV) pseudotype virions. The entire HIV-NL43 gp160 cytoplasmic domain except for three amino acids directly downstream of the transmembrane domain was replaced by the complete RV-G cytoplasmic domain. "p" represents a proline residue not present in the parental proteins. Cytoplasmic and transmembrane domain sequences are separated by a slash (/).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 5

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ACGCTTAACA A 11

(2) INFORMATION FOR SEQ ID NO:2:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AATTCCTGCA GTAATACGAC TCACTATAGG G 31

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Leu Leu Ser Ala Gly Ala Leu Thr Ala Leu Leu Ile Ile Phe Leu Met
1 5 10 15

Thr Cys Cys Arg Arg Val Asn Arg Ser Glu Pro Thr Gln His Asn Leu
20 25 30

Arg Gly Thr Gly Arg
35

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Leu Val Gly Gly Leu Arg Ile Val Phe Ala Val Leu Ser Ile Val Asn
1 5 10 15

Arg Val Arg Pro Arg Arg Val Asn Arg Ser Glu Pro Thr Gln His Asn
20 25 30

Leu Arg Gly Thr Gly Arg
35

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Leu Val Gly Gly Leu Arg Ile Val Phe Ala Val Leu Ser Ile Val Asn
1 5 10 15

Arg Val Arg Gln Gly Tyr Ser Pro Leu Ser Phe Gln Thr His Leu Pro
20 25 30

Ile Pro Arg Gly Pro Asp
35

I claim:

1. A genetically manipulated, infectious, replicating, non-segmented negative-stranded RNA virus mutant, comprising at least one alteration selected from an insertion and a deletion, wherein the alteration is in a region of the virus genome selected from an open reading frame, a pseudogene region and an intergenic region.

2. The virus mutant according to claim **1**, comprising at least one alteration selected from an insertion and a deletion, wherein the alteration is in a pseudogene region.

3. The virus mutant according to claim **1**, comprising at least one alteration selected from an insertion and a deletion, wherein the alteration is in an open reading frame.

4. The virus mutant according to claim **3**, comprising at least one alteration in the open reading frame encoding the Matrix protein or an analog thereof, resulting in the absence of a functional Matrix protein, wherein said mutant can be grown when phenotypically complemented with a functional Matrix protein.

5. The virus mutant according to claim **3**, comprising at least one alteration selected from an insertion and a deletion, wherein the alteration is in the open reading frame encoding the glycoprotein G.

6. The virus mutant according to claim **5**, wherein the alteration results in the absence of a functional glycoprotein G, wherein said mutant can be grown when phenotypically complemented with a functional glycoprotein G analog.

7. The virus mutant according to claim **6**, wherein the glycoprotein G analog is the rabies glycoprotein G.

8. The virus mutant according to claim **1**, comprising a heterologous nucleic acid sequence encoding an epitope or polypeptide of a pathogenic virus or microorganism.

9. The virus mutant according to claim **1**, wherein the virus mutant belongs to the family of paramyxoviridae.

10. The virus mutant according to claim **1**, wherein the virus mutant belongs to the family of rhabdoviridae.

11. The virus mutant according to claim **10**, wherein the virus mutant is a rabies virus.

12. A vaccine for the prevention of infection caused by a non-segmented negative-stranded RNA virus in a mammal, comprising a virus mutant according to claim **1** and a pharmaceutically acceptable carrier or diluent.

13. A process for the preparation of an infectious, replicating, non-segmented, negative-stranded RNA virus comprising the steps of:

a) providing a host cell expressing a heterologous RNA polymerase;

b) introducing into said host cell

1) one or more DNA molecules encoding the virus N, P and L proteins, or analogs thereof operably linked to expression control sequence functional in said host cell; and

2) a DNA molecule comprising the cDNA of the non-segmented negative-stranded RNA virus wherein the cDNA encodes the entire genome of the virus or is modified by the incorporation of a mutation, and wherein the DNA molecule is transcribed by the heterologous RNA polymerase, and

c) isolating the viruses produced by the cells.

14. The process according to claim **13**, wherein the cDNA of the non-segmented negative-stranded RNA virus genome is modified by the incorporation of a mutation.

15. The process according to claim **13**, wherein the transcripts of the non-segmented negative-stranded RNA virus cDNA genome are positive stranded antigenomic RNAs.

16. The process according to claim **13**, wherein the RNA polymerase is T7 RNA polymerase.

17. The process according to claim **13**, wherein the non-segmented negative-stranded RNA virus genome is obtained from the family of paramyxoviridae.

18. The process according to claim **13**, wherein the non-segmented negative-stranded RNA virus genome is obtained from the family of rhabdoviridae.

19. The process according to claim **18**, wherein the non-segmented negative-stranded RNA virus genome is obtained from the rabies virus.

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